Differential thiol oxidation of the signalling proteins Akt, PTEN or PP2A determines whether Akt phosphorylation is enhanced or inhibited by oxidative stress in C2C12 myotubes derived from skeletal muscle.

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ABSTRACT

Oxidative stress, caused by excess reactive oxygen species (ROS), has been hypothesised to cause or exacerbate skeletal muscle wasting in a number of diseases and chronic conditions. ROS, such as hydrogen peroxide, have the potential to affect signal transduction pathways such as the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway that regulates protein synthesis. Previous studies have found contradictory outcomes for the effect of ROS on the PI3K/Akt signalling pathway, where oxidative stress can either enhance or inhibit Akt phosphorylation. The apparent contradictions could reflect differences in experimental cell types or types of ROS treatments. We replicate both effects in myotubes of cultured skeletal muscle C2C12 cells, and show that increased oxidative stress can either inhibit or enhance Akt phosphorylation. This differential response could be explained: thiol oxidation of Akt, but not the phosphatases PTEN or PP2A, caused a decline in Akt phosphorylation; whereas the thiol oxidation of Akt, PTEN and PP2A increased Akt phosphorylation. These observations indicate that a more complete understanding of the effects of oxidative stress on a signal transduction pathway comes not only from identifying the proteins susceptible to thiol oxidation, but also their relative sensitivity to ROS.

1 INTRODUCTION

Skeletal muscle wasting contributes to morbidity and mortality in a number of chronic diseases including aging, diabetes, cachexia, disuse and dystrophy [reviewed in (Alfadda & Sallam, 2012; Doria *et al*, 2012; Kim *et al*, 2013; Moylan & Reid, 2007; Onesti & Guttridge, 2014)]. Oxidative stress, caused by excess reactive oxygen species (ROS), has been hypothesised to be a contributory factor in causing myofiber death, and in decreasing the size of myofibers as the result of loss of protein content (Arthur *et al*, 2008; Jackman & Kandarian, 2004; Lang *et al*, 2010; Moylan & Reid, 2007; Sakuma & Yamaguchi, 2012; Shavlakadze & Grounds, 2006). There are diverse mechanisms by which ROS can affect myofibers because the different forms of ROS have differing chemical properties (Arthur *et al*, 2008; Barbieri & Sestili, 2012). In the context of loss of protein content, ROS such as hydroxyl radicals cause irreversible oxidative damage to proteins which promotes catabolism of damaged proteins (Birben *et al*, 2012; Davies, 1987; Dean *et al*, 1997; Gomes-Marcondes & Tisdale, 2002). Other ROS, such as hydrogen peroxide, can affect protein function through the reversible oxidation of thiol groups. As a consequence, ROS have the potential to affect signal transduction pathways that regulate protein turnover by affecting the actions of proteins such as kinases, phosphatases and transcription factors via reversible protein thiol oxidation (Arthur *et al*, 2008; Muller *et al*, 2006; Szewczyk & Jacobson, 2005).

One way in which ROS can alter protein turnover is by blunting signalling pathways that promote protein synthesis, such as the key PI3K/Akt signalling pathway. Insulin-like growth factor 1 (IGF-1), a potent growth factor which activates the PI3K/Akt signalling pathway, plays a fundamental role in regulating protein synthesis and hypertrophic muscle growth (Glass, 2005; Rommel *et al*, 1999; Shavlakadze & Grounds, 2006). The importance of IGF-1 as a regulator of muscle mass is supported by studies showing that, in the absence of injury in mice, locally synthesised IGF-1 pro-peptide increased muscle mass, strength and resistance to atrophy without changing serum IGF-1 levels (Barton, 2006; Musaro *et al*, 2001; Shavlakadze *et al*, 2010). Given the anabolic effects that it exerts in growing and cultured muscle cells, IGF-1 has been proposed as a therapeutic agent for preventing muscle wasting in a range of situations. PI3K/Akt signalling also represents a major signalling hub between cytokine, growth factor, and integrin signalling pathways crucial to many biological processes including cell growth, survival, proliferation and motility (Leslie, 2006; Taniguchi *et al*, 2006).

Many studies show that ROS can affect the activity of the PI3K/Akt signalling pathway. Some studies demonstrate that ROS inhibit the activation of PI3K/Akt signalling (Berdichevsky *et al*, 2010; Durgadoss *et al*, 2012; Gardner *et al*, 2003; Murata *et al*, 2003) and the proposed mechanism for this is the reversible oxidation of Akt, which lies downstream of PI3K. Akt is activated upon its phosphorylation at Thr308 and Ser473 (Alessi et al, 1996) and the crystal structure of the non-phosphorylated (inactive) Akt2 kinase domain shows that in the activation loop of Akt2, Cys297 can form a disulphide bond with Cys311. The disulphide bond was sensitive to a reducing agent, indicating that oxidation of Akt is reversible (Huang *et al*, 2003). Further work by Murata *et al*. (2003) in rat heart cells showed that the formation of this disulphide bond was induced by hydrogen peroxide treatment. Although oxidation of Akt did not affect its kinase activity, increased interaction between oxidised Akt and phosphatase PP2A was proposed to lead to dephosphorylation of Akt (Murata *et al*, 2003). A recent *in vivo* study supports this contention by demonstrating that oxidation of Akt in mouse midbrain is associated with down-regulation of Akt phosphorylation (Durgadoss *et al*, 2012). Together, this evidence

supports the hypothesis that oxidation of Akt by ROS leads to PI3K/Akt signalling inhibition.

In contrast to studies demonstrating that ROS cause a decline in the PI3K/Akt signalling pathway, there is also evidence that treatment with ROS leads to the activation of PI3K/Akt signalling (Konishi *et al*, 1997; Mackey *et al*, 2008; Sadidi *et al*, 2009; Shaw *et al*, 1998; Ushio-Fukai *et al*, 1999; Wu *et al*, 2012). The mechanism has been proposed to involve the inhibition of PTEN, a phosphatase which plays a key role in decreasing PI3K/Akt signalling [reviewed in (Lacalle *et al*, 2004; Leslie, 2006; Leslie & Downes, 2002; Sulis & Parsons, 2003)]. PTEN contains a highly reactive cysteine residue in the conserved catalytic site that is susceptible to thiol oxidation by ROS [reviewed in (Leslie, 2006)]. Oxidation of the cysteine inactivates the phosphatase, thus preventing it from inhibiting the PI3K/Akt pathway (Lee *et al*, 2002). PP2A, a phosphatase involved in dephosphorylating Akt (Resjo *et al*, 2002), can also be inactivated by ROS (Foley *et al*, 2007; Rao & Clayton, 2002) but whether oxidative inactivation PP2A participates in the activation of Akt signalling has not been examined.

These previous studies clearly present contradictory outcomes for the effect of ROS on the PI3K/Akt signalling pathway, where ROS can either enhance or inhibit the pathway via thiol oxidation of signalling proteins PTEN and Akt. The apparent contradictions could reflect differences in experimental cell types or types of ROS treatments. Alternatively, differences in the sensitivity of protein thiol groups to different levels of ROS could differentially affect Akt phosphorylation. To test this possibility, we manipulated hydrogen peroxide levels in C2C12 myotube cultures (a mouse model for young skeletal myofibres) and examined the effect on Akt phosphorylation in the presence and absence of IGF-1 (to stimulate signalling). Cells in culture are subject to oxidative stress caused by cell culture media and by exposure to higher oxygen concentrations (21%) than *in vivo* oxygen concentrations of about 4-5% in muscle (Halliwell, 2003; Lui *et al*, 2010; Richardson *et al*, 2006). For this reason, we initially investigated the effects of a decrease in hydrogen peroxide levels, using the enzyme catalase which is specific for hydrogen peroxide (Chance *et al*, 1979; Chelikani *et al*, 2004). We then used treatment with hydrogen peroxide or glucose oxidase, an enzyme which generates hydrogen peroxide (Raba & Mottola, 1995), to increase media levels of hydrogen peroxide. The aims of this study were to determine how hydrogen peroxide affects (1) the basal level of Akt phosphorylation, (2) the stimulation of Akt phosphorylation by IGF-1 and, (3) whether hydrogen peroxide could be affecting Akt phosphorylation via reversible thiol oxidation of PTEN, PP2A or Akt.

2 MATERIAL AND METHODS

2.1 C2C12 myotubes

The C2C12 skeletal muscle immortalised mouse cell line was from the American Type Culture Collection (ATCC; Manassas, USA). Undifferentiated myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum at 37°C in the presence of 5% CO_2. The myoblasts were fused into myotubes by changing them into differentiation/fusion medium (DMEM supplemented with 2% horse serum), with change of medium every 48 h. All experiments were conducted using myotubes at 7 days post differentiation.

2.2 Treatments

Long R^3 IGF-1 Recombinant Analog, catalase (Cat), glucose oxidase (GO), diamide, N-acetyl cysteine (NAC) and inhibitors (LY294002 and rapamycin) were from Sigma-Aldrich. Hydrogen peroxide was purchased from BDH laboratories. For low glucose oxidase treatment, serum-starved myotubes were treated overnight (18 h). For catalase and high glucose oxidase treatments, myotubes were serum starved for 30 min and subjected to 1 h treatments. Myotubes treated with diamide, NAC and hydrogen peroxide were serum-starved for 1.5 h and subjected to 10 min treatments. For IGF-1 treatments, IGF-1 was added to myotube cultures 30 min prior to protein extraction for all experiments. Treatments did not cause significant increases in cell death, as measured by lactate dehydrogenase release (see supplementary figure).

2.3 Protein extraction and immunoblotting

Following treatments, 7-day-old myotubes were lysed in five times volume (w/v) of PhosphoSafe Extraction Buffer (Novagen) supplemented with protease inhibitor tablets (Roche), followed by incubation on ice for 20 min. The lysates were centrifuged at 12,000 *g* for 10 min and the supernatant was stored at -80°C until used for analysis. Total protein was quantified using the Bradford assay (Bio-Rad). Protein samples were resolved on 10% TGX gels (Bio-Rad) and protein was transferred onto Nitrocellulose membrane using a Trans Turbo Blot system (Bio-Rad). Western blotting was performed with phospho-Akt (Ser473) (no. 9271), Akt (no. 9272), PTEN (no. 9552), PP2A (no. 2038), all from Cell Signaling. Donkey anti-rabbit HRP-conjugated secondary antibody (Pierce) was used to detect a primary antibody. Chemiluminescence imaging was performed on ChemiDoc MP Imaging System (Bio-Rad).

2.4 Dual fluorescence labelling of protein thiols

Reduced and oxidised protein thiols in myotubes culture were measured using a modification of the dual fluorescence labelling technique, established by Armstrong *et al* (2011). Following treatments, myotubes were lysed in 20% (w/v) trichloroacetic acid (TCA) in acetone to denature and precipitate the protein as well as to avoid any further oxidation reactions. Protein was collected by centrifugation at 10,000 *g* for 5 min at 4°C, washed twice with acetone and solubilised in SDS buffer (0.5% SDS, 0.5 M Tris, pH 7.3). The protein pellet was further solubilised by sonication until completely dispersed and divided into two aliquots. The first aliquot was used for protein quantitation using a Micro BCA assay kit (Sigma-Aldrich). Approximately 100 µg of protein from the second aliquot was used for labelling. Protein was solubilised in 50 µl of SDS buffer (pH 7.0) and first labelled with BODIPY FL-N-(-2-aminoethyl) maleimide (FLM; Invitrogen). Unbound FLM was removed with acetone and incubated overnight at -20°C to precipitate protein. Protein was re-solubilised in SDS buffer and oxidised thiols were reduced with tris(2-carboxyethyl)phosphine (TCEP, Sigma–Aldrich). Unlabelled reduced thiols were labelled with a second fluorescent dye, Texas Red C2-maleimide (Invitrogen). Unbound dye was washed off with acetone and the sample re-suspended in SDS buffer. Samples were read using a fluorescent plate reader (Fluostar Optima) with wavelengths set at excitation 485 nm, emission 520 nm for FLM and at excitation 544 nm, emission 610 nm for Texas Red. Pre-labelled ovalbumin was used to generate a standard curve for each dye and results were expressed per mg of protein, quantified using Detergent Compatible (DC) protein assay (BioRad). The remaining labelled protein samples and pre-labelled ovalbumin standards were run on SDS-PAGE. The actin band was analysed using the ChemiDoc MP Imaging System (Bio-Rad) to determine relative oxidation [oxidised/ (oxidised + reduced)].

2.5 Determination of oxidation states of specific proteins using immunoassay

The dual fluorescence labelling technique did not have sufficient resolution to detect the oxidation state of specific signal transduction proteins. As a consequence, the oxidation states of Akt, PTEN and PP2A were determined using a protocol modified from the dual fluorescence labelling technique (Armstrong *et al*, 2011) and the method developed by Wu *et al* (2000). Protein was collected and labelled as described for the dual fluorescence labelling method, except FLM was replaced with 10 mM N-ethylmaleimide (Sigma) and Texas Red was replaced with 20 mM M-PEG-MAL (5 kDa, Jerkem Technology). Labelled protein was quantified using the DC protein assay, separated by SDS-PAGE and analysed by immunoblotting. The labelling of each protein thiol with M-PEG-MAL resulted in an approximately 5 kDa shift on SDS-PAGE.

2.6 Result analysis

Images from immunoblotting were quantified using Image J software (Schneider *et al*, 2012). The phosphorylation level of Akt was expressed as the densitometry ratio of phosphorylated Akt to total Akt, whereas the oxidation levels of Akt, PTEN and PP2A were expressed as densitometry ratio of oxidised to total Akt, PTEN and PP2A, respectively. Each experiment was repeated three times (N=3) on different days with separate batches of myotube cultures (fused from myoblasts resurrected on different days). In each experimental set, myotube cultures were treated in duplicate (two separate dishes) and the average result was taken. The ratio values of treated samples were normalised to the untreated (UNT) serum-starved samples within each N, whereby the untreated samples were set to a value of 1. For immunoblotting images of Akt phosphorylation experiments, duplicate samples are shown for each treatment. The vertical black lines on immunoblotting images indicate where the same gel was cropped to only show the relevant treatments.

2.7 Statistical analysis

Statistical analyses were performed with GenStat Software 14th Edition. A one-way ANOVA, followed by post-hoc least significant difference (LSD) tests was used to compare values among the treatments. Data were assessed for normal distribution and not normally distributed data were $\log_{10}$ transformed for statistical analysis. Differences were accepted as significant at $P<0.05$. Values in the figures are reported as mean $\pm$ SEM and only differences between groups of interest are indicated.

3 RESULTS

3.1 Effects of catalase and glucose oxidase on Akt phosphorylation

Catalase treatment, to decrease the level of hydrogen peroxide in myotube cultures, increased the basal level of Akt phosphorylation by 231% (Fig. 1A). To examine if a decrease in hydrogen peroxide affected the stimulation of Akt phosphorylation by IGF-1, myotubes were pre-treated with catalase. In the absence of catalase, Akt phosphorylation was increased by 252% following treatment with IGF-1 (Fig. 1A). In the presence of catalase and following stimulation with IGF-1, Akt phosphorylation was 509% higher than untreated cells, which appeared to be an additive effect of increased Akt phosphorylation resulting from IGF-1 stimulation and catalase treatments (Fig. 1A).

Next the effect of an increase in the level of hydrogen peroxide on basal Akt phosphorylation and stimulation by IGF-1 was examined. Low glucose oxidase (LGO)

treatment caused a 37% decrease in the basal level of Akt phosphorylation compared with untreated myotubes (Fig. 1B). Stimulation by IGF-1 increased Akt phosphorylation in the presence of LGO (Fig. 1B; IGF-1 compared with UNT) that was comparable to the absence of LGO (Fig. 1B; LGO+IGF-1 compared with LGO).

Akt can be activated in response to various stimuli via different signalling pathways (Brazil & Hemmings, 2001; Datta *et al*, 1999; Sarbassov *et al*, 2005). mTOR has been suggested to cause phosphorylation of Akt (Sarbassov *et al*, 2005), but rapamycin, an inhibitor of mTOR did not affect the basal level of Akt phosphorylation (Fig. 1C). In contrast, LY294002, a PI3K inhibitor, decreased the basal level of Akt phosphorylation (Fig. 1D) and blocked a 110% increase in Akt phosphorylation caused by catalase treatment alone (Fig. 1E).

Together, these data show that basal Akt phosphorylation is dependent on PI3K, and that there is an inverse relationship between the levels of hydrogen peroxide and the basal level of Akt phosphorylation. However, the changes did not blunt the magnitude of the increase in Akt phosphorylation in response to IGF-1 stimulation.

3.2 Effects of thiol oxidant and reductant on Akt phosphorylation

To test whether changes in protein thiol oxidation could affect Akt phosphorylation, cells were treated with the thiol oxidising agent diamide or the thiol reducing agent NAC. Treatment with diamide decreased the basal level of Akt phosphorylation by 85% compared with controls (Fig. 2A), with IGF-1 stimulated Akt phosphorylation halved following diamide pre-treatment in myotubes (Fig. 2A). These data are consistent with increased protein thiol oxidation mediating Akt phosphorylation by hydrogen peroxide.

The thiol reducing agent NAC did not affect basal levels of Akt phosphorylation (Fig. 2B), but did attenuate the response to IGF-1 (Fig. 2B). This observation contrasts with the catalase results where both basal and IGF-1 stimulated Akt phosphorylation were increased (Fig. 1A). These paradoxical findings are addressed in the discussion.

3.3 Effects of antioxidant and oxidant treatments on Akt and PTEN oxidation

Akt and PTEN have been previously identified as susceptible to oxidation in the PI3/Akt pathway (Huang *et al*, 2003; Leslie, 2006). Therefore, the thiol oxidation states of both molecules were examined using a method based on the labelling of oxidised protein with M-PEG-MAL, which causes mobility shifts of proteins on SDS-PAGE (Wu *et al*, 2000). Catalase treatment caused a 46% decrease in Akt oxidation (Fig. 3A), but did not significantly change PTEN oxidation (Fig. 3B). Increasing hydrogen peroxide, by treatment with low glucose oxidase, caused a 95% increase in Akt oxidation (Fig. 3C), but did not significantly affect PTEN oxidation (Fig. 3D). IGF-1 did not cause significant changes in the oxidation of Akt or PTEN (Fig. 3E, F). These data indicate that Akt is more susceptible to oxidation than PTEN.

The actions of the thiol modifying reagents differed from those of the enzymes (catalase and glucose oxidase) which specifically modulate levels of hydrogen peroxide. NAC did not affect the thiol oxidation state of Akt nor PTEN (Fig. 3G, H), whereas diamide increased thiol oxidation of both Akt and PTEN (Fig. 3G, H). These discrepancies are addressed in the discussion.

3.4 Effects of antioxidant and oxidant treatments on protein thiol oxidation levels

Catalase and low glucose oxidase caused changes in Akt oxidation that were not evident for PTEN, which raised the possibility that Akt was particularly sensitive to changes in the oxidative environment. In this context, neither catalase (Fig. 4A) nor glucose oxidase (Fig. 4B) caused significant changes in global measures of protein thiol oxidation utilising fluorescent dyes (Armstrong *et al*, 2011). We have previously shown that the intracellular protein actin, the monomeric subunit of thin filaments of muscles, is susceptible to thiol oxidation *in vitro* and *in vivo* (Armstrong *et al*, 2011; Terrill *et al*, 2013): however, there was no significant changes in actin thiol oxidation for myotubes treated with catalase or glucose oxidase (Fig. 4C, D). These data for actin emphasise that Akt is particularly sensitive to changes in the oxidative environment.

The effect of treatments with IGF-1, NAC, or diamide on protein thiol oxidation was also examined. Neither IGF-1 nor NAC affected global protein thiol oxidation, whereas diamide caused protein thiol oxidation (Fig. 4A). Actin oxidation was not affected by treatment with NAC, but was substantially increased by diamide (Fig. 4E).

3.5 Effects of high glucose oxidase treatment and hydrogen peroxide level on Akt phosphorylation and oxidation states of Akt, PTEN and PP2A

Previous studies have linked oxidation of PTEN to changes in the activation of Akt [reviewed in (Leslie, 2006)]. However, our data show that the phosphorylation level of Akt was being affected without oxidation of PTEN. We therefore tested whether PTEN oxidation could affect Akt phosphorylation in C2C12 myotubes by treating with 10 times higher activity of glucose oxidase (100 mU/ml). PTEN oxidation was increased by 465% when compared with untreated serum-starved myotubes (Fig. 5A). As a consequence, the basal level of Akt phosphorylation in myotubes treated with high glucose oxidase was increased by 160% when compared with untreated myotubes (Fig. 5B), despite an increase in Akt oxidation (Fig. 5C). In a separate experiment, direct treatment of myotube cultures with hydrogen peroxide, which increased PTEN oxidation by 1183% (Fig. 5D), also increased Akt phosphorylation level by 1064% (Fig. 5E).

In addition to PTEN, other phosphatases are sensitive to oxidative stress. PP2A, the phosphatase responsible for dephosphorylation of phosphorylated Akt can be oxidised by ROS, leading to inhibition of its phosphatase activity (Finnegan *et al*, 2010; Foley *et al*, 2007; Rao & Clayton, 2002; Sen *et al*, 2012). Therefore, the thiol oxidation state of PP2A was also examined to determine if PP2A was sensitive to oxidation by glucose oxidase and hydrogen peroxide. In the presence of low glucose oxidase treatment, there was no evidence of increased oxidation of PP2A (Fig. 6A). However, high glucose oxidase or hydrogen peroxide increased the oxidation of PP2A, by 247% and 768% respectively (Fig. 6B, C).

4 DISCUSSION

The primary finding of this study is that increased oxidative stress can, in an apparent paradox, either enhance or inhibit Akt phosphorylation in (7-day-old) C2C12 myotube cultures. Our findings bring together previous findings whereby increased oxidative stress enhanced Akt phosphorylation or alternatively, in separate experimental models, inhibited Akt phosphorylation (Berdichevsky *et al*, 2010; Durgadoss *et al*, 2012; Gardner *et al*, 2003;

Konishi *et al*, 1997; Mackey *et al*, 2008; Murata *et al*, 2003; Sadidi *et al*, 2009; Shaw *et al*, 1998; Ushio-Fukai *et al*, 1999; Wu *et al*, 2012).

In part, our observations are consistent with previous studies where increased hydrogen peroxide decreased Akt phosphorylation (Berdichevsky *et al*, 2010; Gardner *et al*, 2003): the mechanism was ascribed to increased dephosphorylation of Akt caused by increased interaction between PP2A and oxidised Akt (Durgadoss *et al*, 2012; Murata *et al*, 2003). Our data with catalase and low glucose oxidase are consistent with this mechanism. Our studies also indicate that it is unlikely that other components of the PI3K/Akt signalling pathway were affected, since Akt phosphorylation was equally responsive to stimulation by IGF-1 in the presence and absence of catalase and low glucose oxidase. In addition, neither catalase nor low glucose oxidase affected the oxidation states of two other redox sensitive proteins, PTEN and PP2A, which had the potential to affect the phosphorylation of Akt.

In contrast to the lack of oxidation of PTEN and PP2A at lower levels of hydrogen peroxide, we found that increasing levels of hydrogen peroxide (using hydrogen peroxide and high glucose oxidase) caused oxidation of both PTEN and PP2A. PTEN contains a reactive cysteine residue that is susceptible to thiol oxidation by ROS [reviewed in (Leslie, 2006)]. Exposure of PTEN to hydrogen peroxide *in vitro* results in oxidation of PTEN with decreased activity (Lee *et al*, 2002). Since PTEN acts as a negative regulator of the PI3K/Akt pathway (Leslie *et al*, 2003), inactivation of PTEN promotes Akt phosphorylation. PP2A, a phosphatase that dephosphorylates Akt, can also be oxidised by ROS, subsequently leading to inhibition of its activity (Finnegan *et al*, 2010; Foley *et al*, 2007; Rao & Clayton, 2002; Sen *et al*, 2012). Previous work has shown PP2A inhibition by hydrogen peroxide in Caco-2 (Rao & Clayton, 2002) and neutroblastoma cell lines (Sommer *et al*, 2002). Another study reported that cadmium-induced ROS inhibited PP2A, leading to the activation of Erk1/2 and JNK (Chen *et al*, 2008). Inhibition of PP2A activity by ROS would promote Akt phosphorylation. Together, inhibition of PTEN and PP2A would act to promote Akt phosphorylation.

We propose that it is the differing sensitivities of Akt, PTEN and PP2A to oxidative stress that provide the answer to the apparent paradox of increased oxidative stress causing both decreased and increased Akt phosphorylation. Low levels of oxidative stress can cause oxidation of Akt to promote dephosphorylation of Akt by PP2A, whereas at higher levels of oxidative stress, inhibition of PTEN and PP2A act to increase Akt phosphorylation (Fig. 7).

Diamide and NAC are commonly used to study the role of ROS in signalling pathways (Anastasiou *et al*, 2011; Handayaningsih *et al*, 2011; Pantano *et al*, 2006; Yeh *et al*, 2007). However, the underlying assumption that thiol oxidant and reductant replicate the effects of changes in ROS may not be valid. For example, diamide, high glucose oxidase and hydrogen peroxide all increased PTEN and PP2A oxidation. However, diamide decreased Akt phosphorylation, whereas high glucose oxidase and hydrogen peroxide increased Akt phosphorylation. It was notable that diamide caused substantial global protein thiol oxidation and actin oxidation, which was not evident with glucose oxidase. This would imply that diamide is causing the oxidation of additional proteins. For NAC, no effect on basal Akt phosphorylation was observed but there was decreased sensitivity of Akt phosphorylation to stimulation by IGF-1, an effect not observed with catalase. As a consequence of these observations, it is apparent that caution is required in interpreting experiments using oxidant and antioxidant treatments which do not specifically target hydrogen peroxide.

Our observations have implications for understanding how signal transduction pathways respond to ROS. First, our data show that hydrogen peroxide can affect multiple protein targets in a signal transduction pathway to affect the outcome of Akt phosphorylation. Second, the protein targets (PTEN, PP2A and Akt) were differentially sensitive to hydrogen peroxide. This concept of signalling proteins having differential sensitivity to thiol oxidation by hydrogen peroxide also helps explain the contradictory findings in the literature. As a consequence, a more complete understanding of the effects of hydrogen peroxide on a signal transduction pathway would not only require identifying the proteins susceptible to thiol oxidation, but also their relative sensitivity to hydrogen peroxide.

Our data indicate that Akt is more sensitive to oxidation than PTEN or PP2A, which likely reflects the effect of the local microenvironment on protein thiol groups (Winterbourn & Hampton, 2008). This differential sensitivity to hydrogen peroxide has implications for understanding how oxidative stress affects cell function in muscles. For example, low levels of chronic oxidative stress which cause Akt oxidation, but not PTEN or PP2A oxidation, can be expected to promote loss of muscle mass. Counterintuitively, higher levels of oxidative stress, by blocking PTEN and PP2A activity, might be expected to promote protein synthesis. Whether this can occur in muscle *in vitro* and *in vivo* will require more information regarding precisely which proteins are oxidised in response to oxidative stress changes in complex physiological environments.

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FIGURE CAPTIONS

Fig. 1
Effects of modulating hydrogen peroxide levels, using [A] catalase (Cat) or [B] low glucose oxidase (LGO) treatments, on phosphorylation of Akt (p-Akt). Myotube cultures were treated with: [A] IGF-1 (10 ng/ml), catalase (500 U/ml) or a combined treatment (IGF-1 + Cat); [B] LGO (10 mU/ml), IGF-1 (30 ng/ml) or a combined treatment (LGO + IGF-1). Effect on phosphorylation of Akt following treatment with: [C] rapamycin (Rap) (50 nM); [D] LY294002 (LY) (50 μM); or [E] combined treatment (LY + Cat). UNT represents untreated myotube cultures. Densitometric quantification of p-Akt to total Akt (t-Akt) is based on n=3 (see 2.6 for detailed description) for each treatment. Bars show statistical difference at *P<0.05* between comparisons. All values are shown as mean ± SEM. For gels, vertical black lines indicate cropping of the same gel to only show the relevant treatments with the centre horizontal black lines separating different gels.

Fig. 2
Effects of manipulating protein thiol oxidation level using [A] diamide and [B] NAC on phosphorylation of Akt (p-Akt)**.** Myotube cultures were treated with [A] diamide (15 mM) or [B] NAC (20 mM) in the presence or absence of IGF-1 (10 ng/ml). UNT represents untreated myotube cultures. Result presentation and statistical analysis are as described in Figure 1.

Fig. 3
Oxidation states of Akt and PTEN following the modulation of hydrogen peroxide level and protein thiol oxidation level. Myotube cultures were treated with: [A][B], Cat (3000 U/ml); [C][D], LGO (10 mU/ml); [E][F], IGF-1 (10 ng/ml); [G][H], NAC (20 mM) or diamide (15 mM). UNT represents untreated myotube cultures. Oxidation states of Akt and PTEN were determined using methoxy PEG-maleimde as described in material and methods. Densitometric quantification is based on n=3 (see 2.6 for detailed description) for each treatment. Horizontal lines show statistical difference at *P<0.05* between comparisons. All values are shown as mean ± SEM. Vertical black lines indicate cropping to only show the relevant treatments.

Fig. 4
[A] Levels of global protein thiol oxidation measured using dual fluorescent in myotube cultures without serum starvation (+serum) or in serum starved and treated with: IGF-1 (10 ng/ml), Cat (3000 U/ml), NAC (20 mM) and diamide (15 mM). [B] Effect of LGO (10 mU/ml) on serum starved myotube cultures. Actin oxidation was measured in serum-starved myotube cultures treated with: [C] Cat (3000 U/ml); [D] LGO (10 mU/ml); [E] NAC (20 mM) and diamide (15 mM). Bars show statistical difference at *P<0.05* relative to untreated myotube cultures (UNT). All values are shown as mean ± SEM.

Fig. 5
Effects of treatment with high GO (100 mU/ml) on [A] PTEN oxidation, [B] Akt phosphorylation and [C] Akt oxidation. Effects of hydrogen peroxide (H_2O_2) (2.5 mM) treatment on [D] PTEN oxidation, [E] Akt phosphorylation and [F] Akt oxidation. Bars show statistical difference at *P<0.05* relative to untreated myotube cultures (UNT). All values are shown as mean ± SEM. Vertical black lines indicate cropping of the same gel to only show the relevant treatments. Horizontal black lines indicate different gels.

Fig. 6
Oxidation of PP2A following the modulation of hydrogen peroxide (H_2O_2) level. Myotube cultures were treated with: [A] LGO (10 mU/ml), [B] high GO (100 mU/ml) and [C] H_2O_2 (2.5 mM). Bars show statistical difference at *P<0.05* relative to untreated myotube cultures (UNT). All values are shown as mean ± SEM. Vertical black lines indicate cropping of the same gel to only show the relevant treatments.

Fig. 7
Scheme depicting how changes in protein thiol oxidation, via altered hydrogen peroxide (H_2O_2) levels, cause different outcomes for the basal level of Akt phosphorylation (p-Akt). In untreated myotubes **(A)**, Akt is partially oxidised, so treatment with **(B)** catalase (to decrease H_2O_2 and oxidative stress) reduces Akt oxidation leading to decreased interaction (and dephosphorylation) with PP2A and hence increased Akt phosphorylation. Mildly increased H_2O_2 by treatment with **(C)** low glucose oxidase (Low GO) does not cause oxidation of PTEN or PP2A but does increase oxidation of Akt, leading to increased interaction (and dephosphorylation) with PP2A and hence decreased Akt phosphorylation. Further increasing levels H_2O_2 by treatment with **(D)** H_2O_2 or high glucose oxidase (High GO) also causes oxidation of PTEN and PP2A, which blocks their activity and leads to increased Akt phosphorylation.

Supplementary
Measurement of extracellular LDH activities in myotube cultures without serum starvation (+serum), serum starved untreated (UNT) or serum starved treated with: [A] IGF-1 (10 ng/ml), Cat (3000 U/ml), NAC (20 mM) and diamide (15 mM); [B] LGO (10 mU/ml); [C] high GO (100 mU/ml) and H_2O_2 (2.5 mM). Culture media from each treatment was collected and assayed for LDH activities. In brief the enzyme activity of LDH released into the culture medium was determined by measuring the rate of decrease of NADH spectrophotometrically at 340 nm (Wolterbeek & van der Meer, 2005). Activity was expressed as LDH activity (Units) over total protein (mg) quantitated using standard Micro Bicinchoninic Acid (BCA) assay (Sigma-Aldrich). Treated myotube cultures were compared to that of serum starved untreated cultures (UNT). UV treated myotube cultures served as a positive control for increased LDH activity due to the loss of cell membrane integrity, whereas myotube cultures without serum starvation (+serum) indicate basal levels of LDH release. Bars show statistical difference at *P<0.05*. All values are shown as mean ± SEM.

Figure(s)

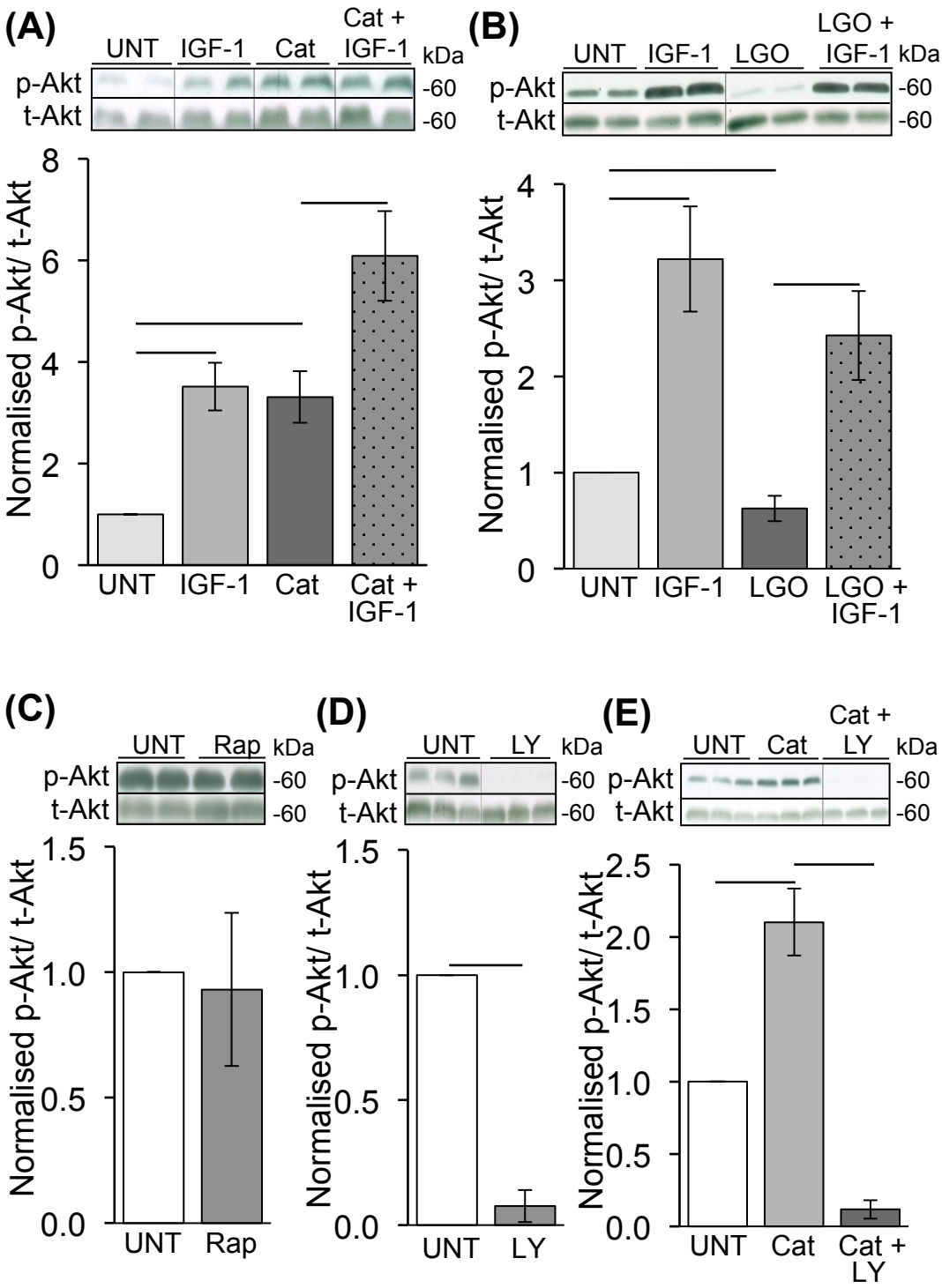

Fig. 1

Figure(s)

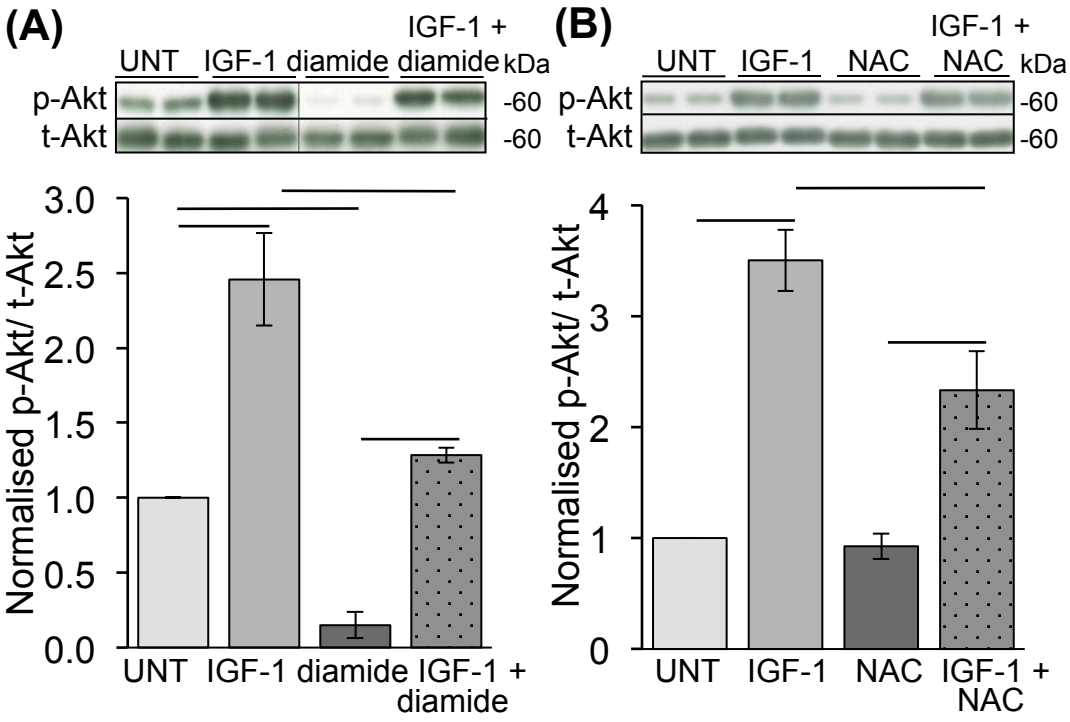

Fig. 2

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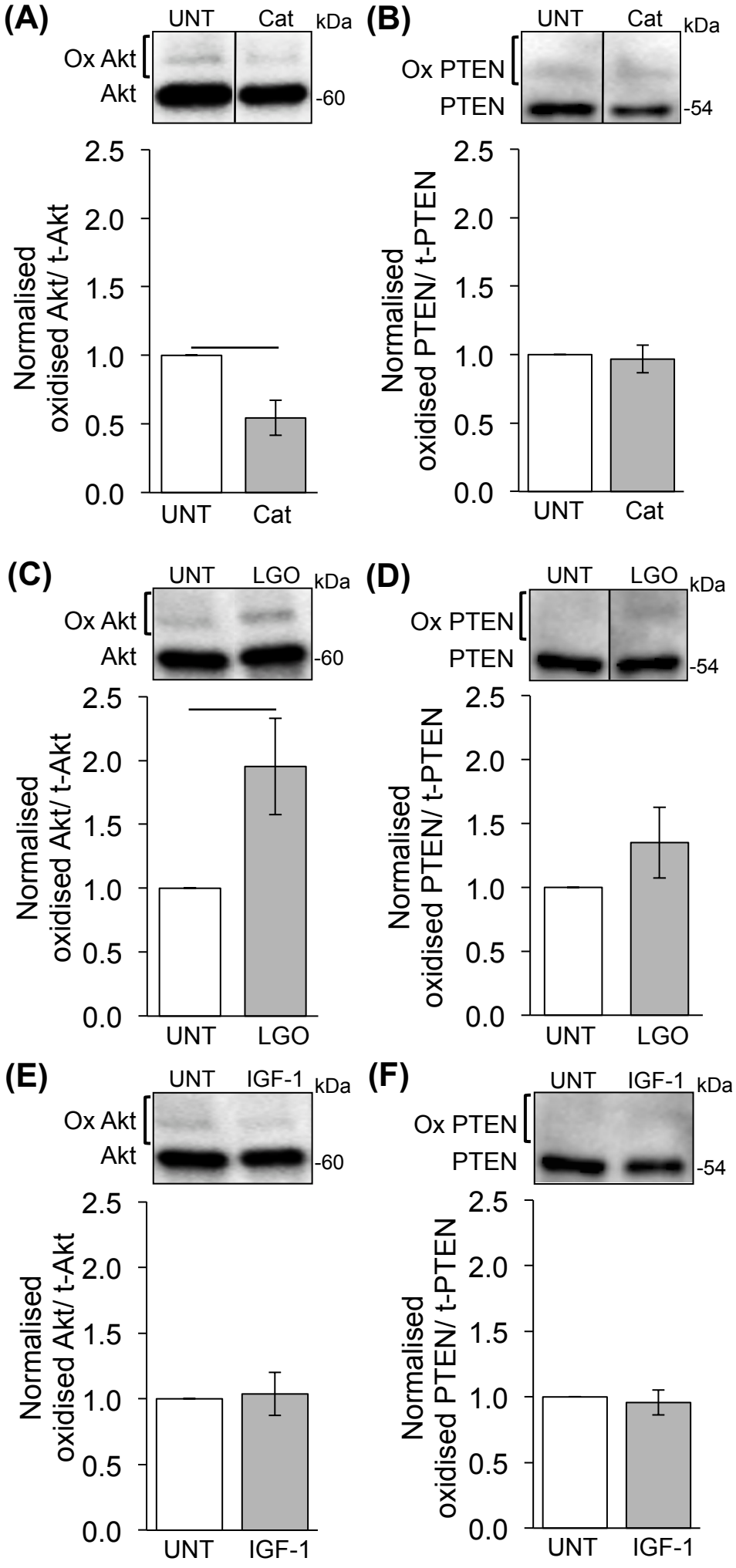
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Ox Akt
Akt
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Normalised oxidised Akt/ t-Akt
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UNT
Cat
(B)
UNT
Cat
kDa
Ox PTEN
PTEN
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2.5
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Ox PTEN
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Normalised oxidised PTEN/ t-PTEN
2.5
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Ox Akt
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Normalised oxidised Akt/ t-Akt
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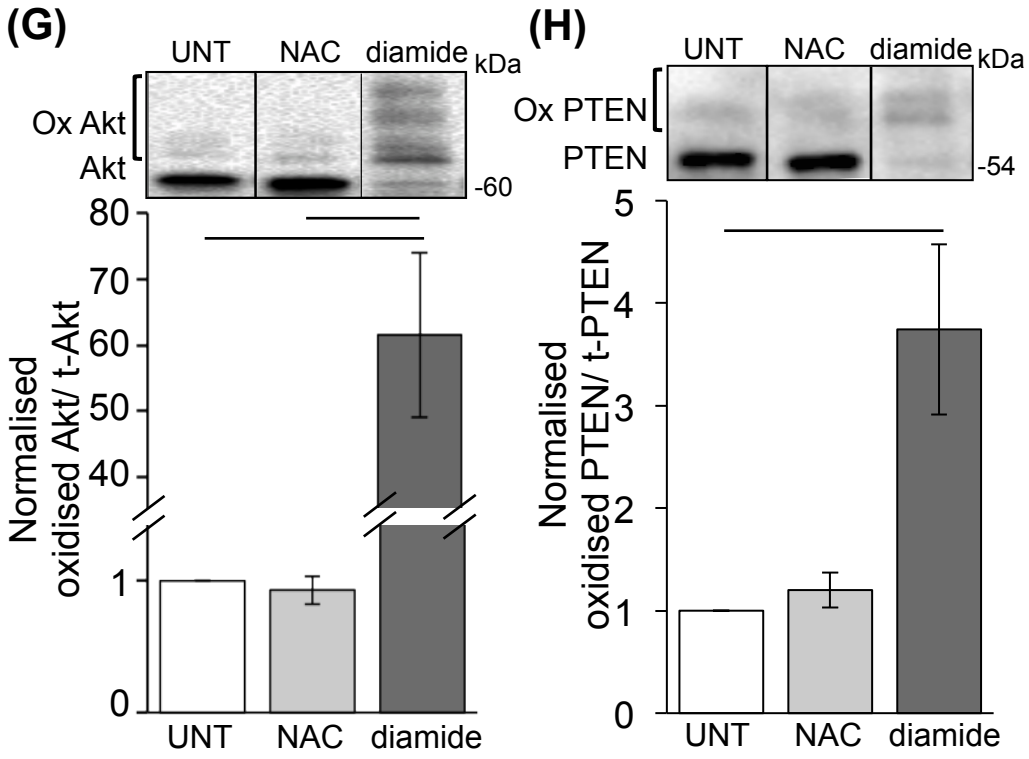
(G)
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UNT
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NAC
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Fig. 3

Figure(s)

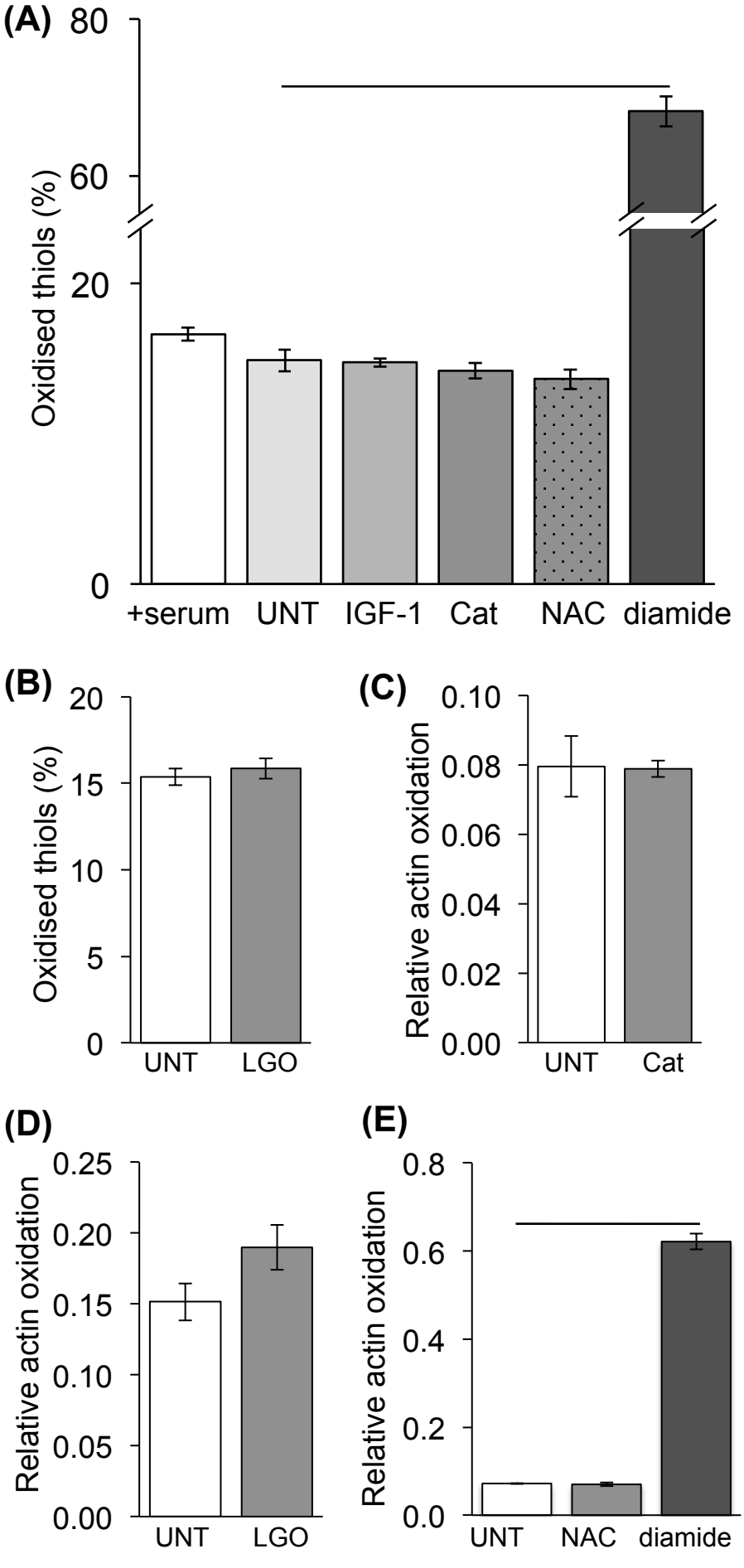

Fig. 4

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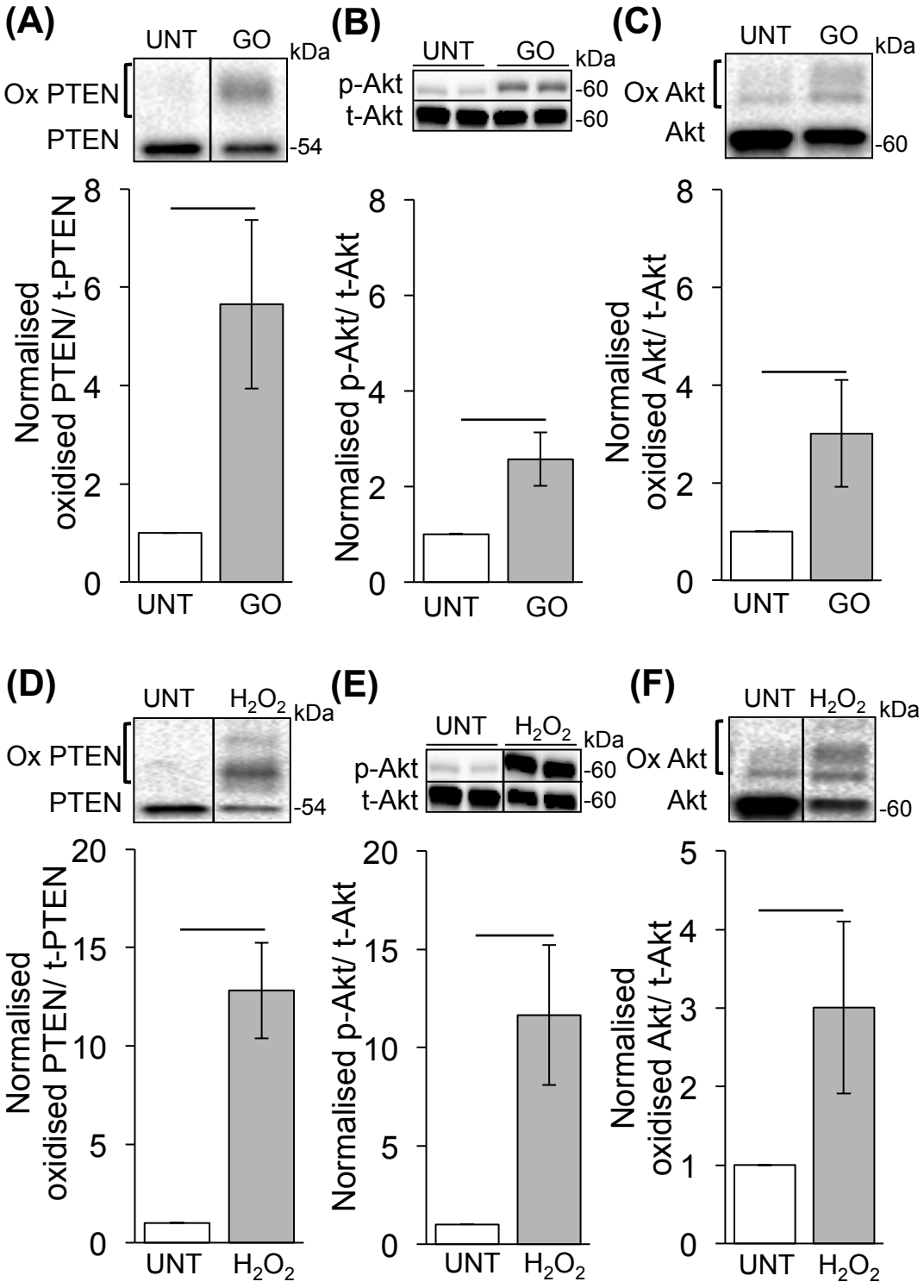

Fig. 5

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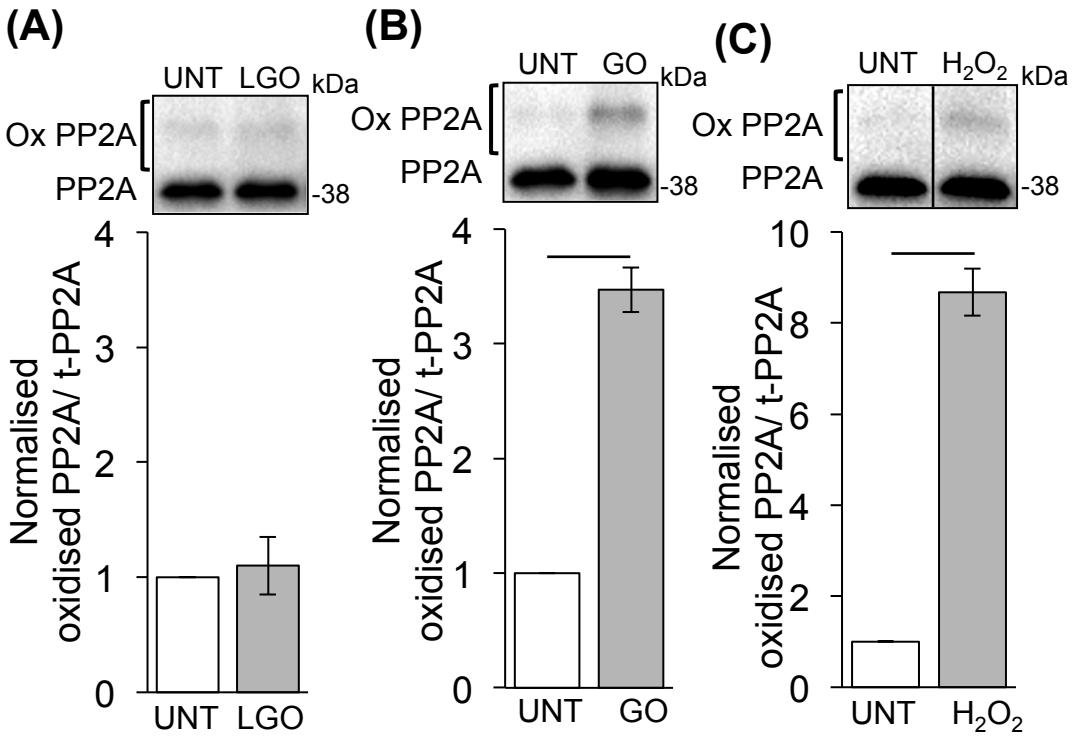

Fig. 6

Figure(s)

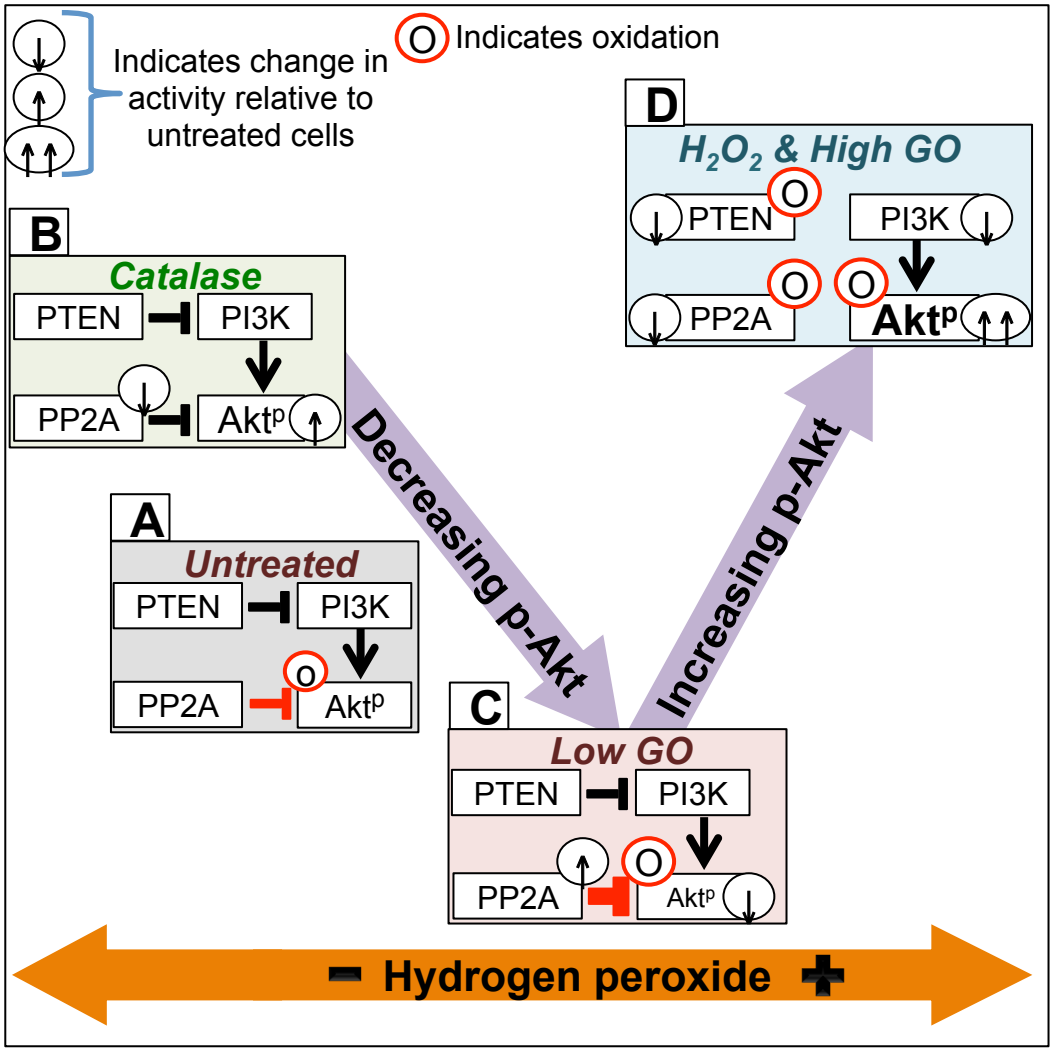

Fig. 7

Supplementary Files

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