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Research Paper

COPPER INDUCED OXIDATIVE STRESS MODULATE ACTIVITIES OF CA^{2+} – DEPENDENT ENZYMES IN *NEUROSPORA CRASSA*

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Employing the well-studied model of copper induced oxidative stress in Ascomycete fungus, *Neurospora crassa*, the present investigation was conducted to ascertain the correlations between copper induced oxidative stress and Ca^{2+}-homeostasis. Transient exposure of a wild strain of *N. crassa* to 0.5 mM copper lead to increased reactive oxygen species, lipid peroxidation, activities of antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase and decreased the ratio of reduced to oxidized glutathione ($p < 0.05$). Presence of 5 mM H_2O_2 in culture media enhanced the copper induced oxidative stress conditions as well as increased intracellular Ca^{2+} levels that could be attenuated by antioxidant, 10 mM N-acetyl cysteine (NAC). Copper induced stress caused decrease in the activities of Ca^{2+}-dependent protein kinase C (PKC) and calcineurin ($p < 0.05$). On the contrary, there was no change in calmodulin and calcineurin contents and the activity of Ca^{2+}-calmodulin dependent protein kinase II in response to copper. Those changes induced by copper enhanced by H_2O_2 and attenuated with NAC. In light of these observations it may suggest that oxidative stress is indeed associated with significant alterations of Ca^{2+}-signaling enzymes involved in regulatory pathways in this lower eukaryote.

Keywords: Calcineurin, Glutathione, Metallothionein, Protein kinase C, Reactive oxygen species

INTRODUCTION

Metal induced toxicity leads to the generation of deleterious free radicals participating in the oxidative deteriorations of biological macromolecules and altered sulfhydryl homeostasis in a variety of organisms (Fraga, 2005; Jamova and Valko, 2011; Malik and Bharti, 2011). Like other metals, toxic concentrations of copper are also alter cellular redox status because Cu^+ generates Reactive Oxygen Species (ROS) via Fenton reactions (Barbusinski, 2009). Even though, copper is an essential transition metal and playing a key role in metabolic and biosynthetic process, it caused oxidative stress conditions when the organism exposed to elevated levels of copper (Yasokawa

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et al., 2008; Contreras *et al*., 2009). The inherited autosomal recessive disorder, Wilson disease as well as Menke's disease are associated with copper toxicity in humans (Bull *et al*., 1993; Uriu-Adams and Keen, 2005). In addition, copper homeostasis disruption has been found to play a key role in the etiology of neurological disorders such as Alzheimer's disease and Parkinson's disease (Bush and Curtain, 2008; Hung *et al*., 2010). Furthermore, copper at higher concentrations induced copper metallothionein (CuMT) synthesis in *Neurospora crassa* (Munger *et al.,* 1987). CuMT (2.2 kDa) is cysteine (7 residues) rich 26 amino acid containing protein, capable of scavenging 6 molecules of copper with antioxidant property (Cai *et al*., 2005). Subsequent studies revealed that CuMT gene expression in *N. Crassa* induced only under toxic concentrations of copper, but not with H_2O_2 (Kumar *et al*., 2005).

Further, investigations with eukaryotes have earlier indicated that ROS generated through copper toxicity might be stimulating release of Ca^{2+} from intracellular stores in yeasts (Rosenfeld *et al.*, 2010), animals (Suntres and Lui, 2006; Manzl *et al.*, 2004) and activity of Ca^{2+}-dependent protein kinases in plants (Gonalez *et al*., 2010). Further, role of ROS and Ca^{2+} in mediating various signal transduction pathways previously demonstrated using inhibitors of protein kinases and phosphatases (Leonard *et al*., 2004; Mattie *et al*., 2008). Since Ca^{2+} acts as second messenger, fluctuations of Ca^{2+} levels may have a downstream effect upon regulatory components such as calmodulin, PKC, Ca^{2+}/calmodulin-dependent protein kinase II and Ca^{2+}/calmodulin-dependent protein phosphatase 2B, calcineurin resulted in altered gene expression (Ermak and Davies, 2002; Musson and Smit, 2011). Consequently studies on calcium ionophores (A23187), calmodulin antagonists (trifluoperazine, W7, calmidazolium), PKC inhibitor (H7) in rodents and cell lines altered MT gene expression (Adams *et al*., 2002, Gunther *et al*., 2012) indicating the existence of Ca^{2+}-calmodulin mediated regulation of MT gene expression (Saydam *et al*., 2002). Further, bioinformatic approaches have suggested that the presence of Calcineurin Dependent Responsive Element (CDRE) along with regulatory elements such as Antioxidant Response Element (ARE) and Metal Response Elements (MRE) in the -3730 bp upstream regions of CuMT gene in *N. Crassa* (Andrews, 2000; Kumar *et al*., 2005). In addition, regulatory subunit of calcineurin recognized as a CDRE binding protein and playing a putative role in regulation of CuMT gene expression (Kumar *et al*., 2006). Despite such prior knowledge, the role of copper induced oxidative stress in Ca^{2+}-signaling enzymes in *N. crassa* is not yet known. Hence, the present study was conducted to elucidate the copper induced oxidative stress conditions and specific events of altered Ca^{2+}-homeostasis. Results presented herein indicated that copper induced oxidative stress associated with Ca^{2+}-signaling events in this lower eukaryote.

MATERIALS AND METHODS

Organism and Growth Conditions

A wild strain of *Neurospora crassa* (FGSC # 4200) (Fungal Genetics Stock Center, Kansas Univ., USA) was cultured in 10 mL of 0.5x Vogel's medium at 100 rpm for 48 h at 28 ± 1ºC. Copper (0.5 mM, as $CuSO_4$ $5H_2O$) was included aseptically after 48 h of growth to the medium to induce metallothionein synthesis. Either pro-oxidant (5 mM H_2O_2) or antioxidant (10 mM N-

acetyl cysteine; NAC) was included in the medium 2 h before addition of copper.

Warburg Respirometry

Oxygen consumption and carbon dioxide liberation by *N. crassa* grown in presence and absence copper were monitored in a Warburg manometer as described by Jayashree and Subramanyam (2000). Respiratory Quotient (RQ) was calculated as the ratio of the volume of CO_2 liberated to that of O_2 consumed.

Measurement of Reactive Oxygen Species

Mycelia (2 mg) grown under various conditions were incubated with 10 mM DCFDA (dissolved in 0.01% DMSO) in phosphate buffered saline pH 7.4 (PBS) for 20 min at 28°C ± 1 in the dark (Davidson, 1996). Release of the fluorescent dichlorofluorescein upon reaction with ROS was monitored by steady state fluorimetry (Ex/Em = 490/520 nm) using steady state spectrofluorimeter (Perkin Elmer spectrofluorimeter LS-50B, Boston, USA).

Estimation of Metabolites and Antioxidant Enzymes

Cytosolic extracts were prepared by homogenization of the mycelia in liquid nitrogen followed by centrifugation at 15000 ×g for 30 min at 4°C in appropriate buffers (Jayashree and Subramanyam, 2000) and protein content in the extracts was determined (Bradford, 1979). Products of lipid peroxidation were measured as thiobarbituric acid reactive substances (TBARS) employing malondialdehyde as the reference standard (Ernster and Nordenbrand, 1967). Reduced (GSH) and oxidized (GSSG) forms of glutathione concentrations were determined by measuring the fluorescence upon reaction o-phthalaldehyde at Ex/Em = 350/420 nm (Hissin and Hilf, 1976). Glutathione peroxidase (GPx) activity in the microsomes was assayed by estimating the content of GSSG released by the action of GPx (Martinez *et al*., 1979). Super-Oxide Dismutase (SOD) activity was assayed by measuring the ability of the enzyme to inhibit super-oxide anion-dependent auto oxidation of pyrogallol (Marklund and Marklund, 1974). Catalase activity was assayed by determining the rate of decomposition of H_2O_2 (Aebi, 1984).

Measurement of Intracellular Ca^{2+} and Assay of Ca^{2+} Signaling Enzymes

Mycelia (20 mg) were washed with PBS and incubated with 10 µM calcium crimson (Molecular Probes, Eugene, USA) in PBS for 30 min at 37 °C ± 1 in the dark (Haugland, 1997). Fluorescence intensity of Ca^{2+}-bound dye was measured at Ex/Em = 590/615 nm in spectrofluorimeter employing the equation: $[Ca^{2+}]$ free = K_d $[F-F_{min}/F_{max}-F]$, where K_d=187 nM for calcium crimson, F is the fluorescence of the calcium crimson at experimental calcium levels, F_{min} is the fluorescence in presence of 1 mM EGTA, F_{max} is fluorescence in presence of 4 mM $CaCl_2$.

Protein kinase C (PKC) activity assayed in reaction mixtures (100 µl) containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 80 µg histone III-S, 12.5 µM [γ-^{32}P]-ATP (2 mCi/µmol), 4 µg leupeptin, 10 µL lipid micelles (445 µM phosphatidyl serine, 100 µM phorbol 12,13-dibutyrate), 10 mM free Ca^{2+} and enzyme extract (50 µg protein) (Huang *et al*., 1988). Simultaneous assays were performed in presence of 1 mM EGTA to differentiate between Ca^{2+}-dependent and independent activities of PKC.

Ca^{2+}-calmodulin-dependent protein kinase II (CaMK II) activity assayed in a reaction mixture (50 µL) containing 50 mM Pipes, pH 7.0, 10 mM

$MgCl_2$, 0.4 mM $CaCl_2$, 5 µg/µL calmodulin, 1 mg/mL BSA, 10 µM syntide 2, 20 µM [γ-32P]-ATP (2 mCi/µmol) and enzyme extract (50 µg protein) (Hanson *et al*., 1989). Radioactivity incorporated in presence of 5 µM calmidazolium considered as blank.

For assaying the activities of Total Protein Phosphatase (TPP) as well as of calcineurin, mycelial extracts were made by centrifuging (20,000 ×g, 30 min, at 4°C) the homogenates made in a buffer containing Tris (50 mM pH 7.8), 3 mM $MgSO_4 7H_2O$, 1 mM EGTA, 0.5 mM DTT, 0.02% sodium azide, 0.1 mM PMSF. TPP activity was assayed according to Wang and Pallen (1983) employing p-nitrophenyl phosphate as substrate. Calcineurin specific activity was assayed by measuring phosphate released from the RII phosphopeptide using malachite green employing a commercial kit (Calbiochem, CA, USA). Calcineurin and calmodulin contents in the extracts were determined by competitive ELISA method by employing respective monoclonal antibodies (Sigma, St. Louis, USA) (Padma and Subramanyam, 1999).

Statistics

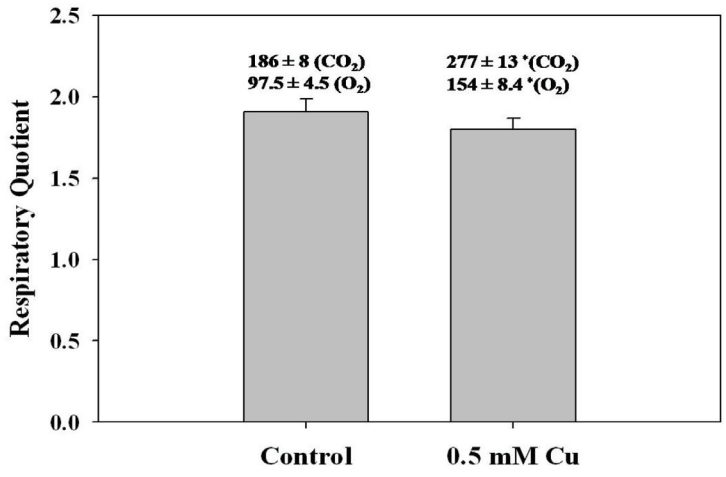
Data were expressed as Mean ± SD obtained from three independent experiments. Statistical significance of obtained results was verified using Student's t test (Sigma Plot 11.0). The criteria for statistical significance were $p < 0.05$.

RESULTS

Copper Induced Stress Enhanced the Oxidative Stress in *N. crassa*

Transient exposure of *N. crassa* to copper enhanced the metabolic rate without changing RQ by increased overall consumption of oxygen and liberation of CO_2 accounting to 52% and 58% increases respectively (Figure 1). Further, copper

Figure 1: Influence of copper induced stress on respiratory quotient in *Neurospora crassa*. *N. crassa* was grown in Vogel medium containing 0.5 mM Cu^{2+} added at 48th hour and harvested at 49th hour. The mycelial pads (1 cm diameter) of equal wet weights were initially equilibrated at 27 ºC for 10 min in 2 ml of culture medium devoid of glucose. Oxygen consumption (O_2) was monitored over a 60 min period in presence of 1 M glucose. Release of CO_2 in presence and absence of 2% KOH was measured. Respiratory quotient (RQ) was calculated as the ratio of the volume of CO_2 liberated to that of O_2 consumed. *p<0.05 in comparison to controls as obtained from three independent experiments



toxicity significantly ($p < 0.01$) enhanced ROS (67%) (Figure 2), TBARS (90%), decreased GSH/GSSG (59%) and increased SOD (103%), catalase (38%) and GPx (21%) activities respectively as compared to untreated controls. Pre-incubation with H_2O_2 alone also caused increase in ROS (14%) and TBARS (27%); decrease in GSH/GSSG (30%) and increase in activities of SOD (62%), catalase (20%) and GPx (11%). However, pre-incubation with H_2O_2 followed by addition of copper *N. crassa* cultures enhanced the ROS production (96%; $p < 0.005$) as well as lipid peroxidation (156%; $p < 0.01$) and activities of SOD (131%; $p < 0.005$), catalase (67%; $p < 0.01$) and GPx (53%; $p < 0.01$) but lessen the GSH/GSSG (70%; $p < 0.005$) when compared to untreated controls. In contrast, pre-incubation of the organism with NAC, resulted in decreased ROS production (25%) and attenuated the copper-induced oxidative stress conditions (Table 1).

Copper Induced Stress Caused Alterations in Ca^{2+} Signaling Enzymes

Transient exposure of *N. crassa* to copper resulted in increased intracellular Ca^{2+} levels (43%) as opposed to 16% increase caused by H_2O_2 as compared to untreated controls (Figure 3). Pre-incubation of cultures with H_2O_2 followed by exposure to copper resulted in further increase in Ca^{2+} levels and accounted to 75 nM. In contrast, NAC did not alter the Ca^{2+} levels but alleviated the copper mediated increase in Ca^{2+} levels. Copper induced stress as wells as H_2O_2 or NAC did not show significant alterations in intracellular calmodulin and calcineurin contents, accounted ~65 and 110 ng/mg protein respectively (data not shown). Likewise, CaMK II activity did not alter either in presence of copper or H_2O_2 or NAC.

Figure 2: Influence of copper induced stress on ROS accumulation. ROS levels in mycelia was monitored by steady state spectrofluorometry ($\lambda_{Ex}/\lambda_{Em}$ = 490/520 nm) upon formation of the fluorescent dichlorofluorescein and depicted as relative fluorescent intensity (RFI) per mg mycelial growth. *$p<0.005$ in comparison to controls as obtained from three independent experiments

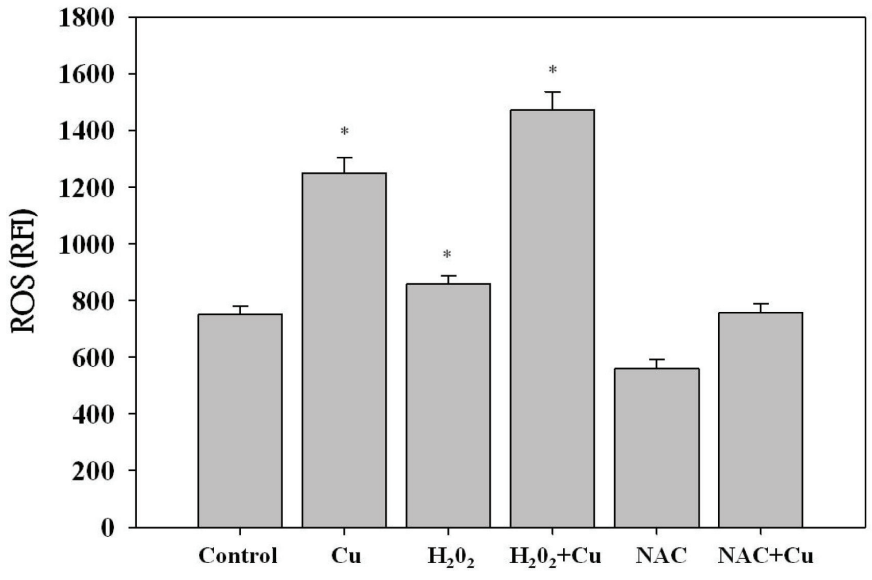
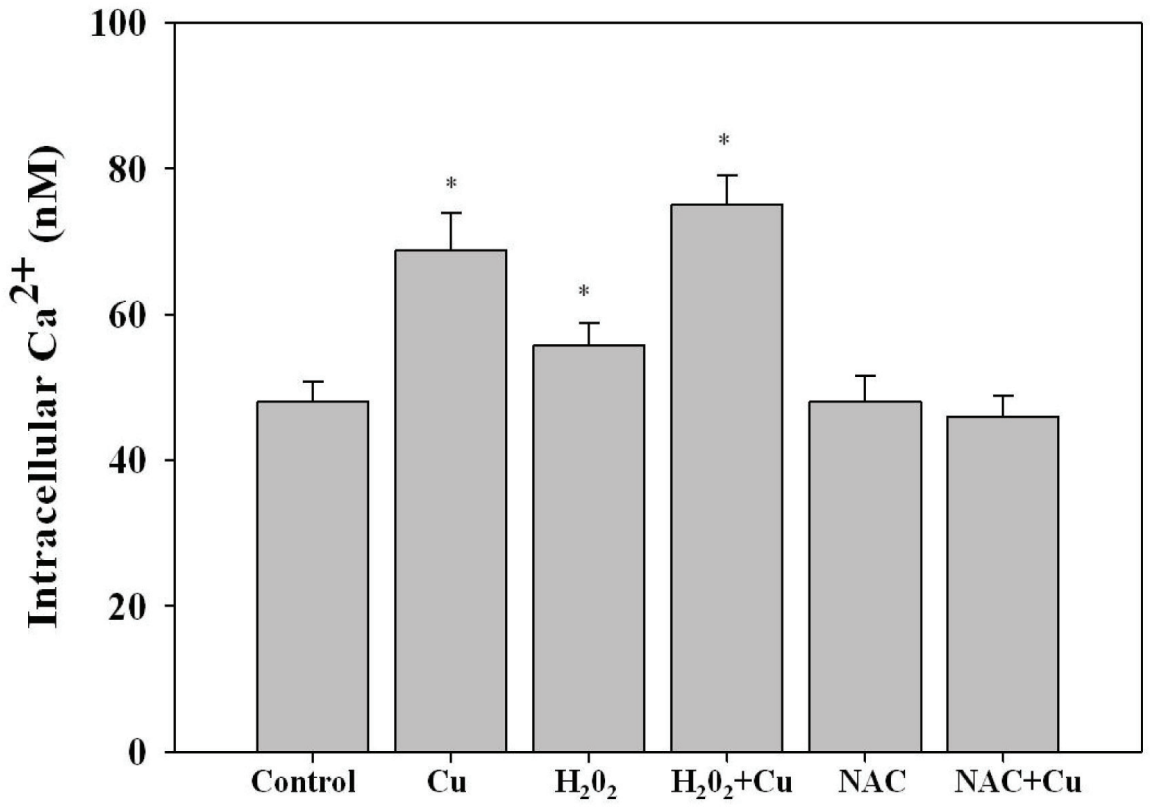


Table 1: Influence of Copper Induced Stress on Oxidative Stress Indicators

Stress Conditions	TBARS (nmoles/min/mg protein)	GSH/GSSG	SOD(Units)	Catalase(Units)	GPx(Units)
Control	82 ± 4.3	2.69 ± 0.15	0.418 ± 0.01	0.55 ± 0.05	26.15 ± 0.6
0.5 mM Cu	156 ± 7.3*	1.13 ± 0.1*	0.852 ± 0.03*	0.76 ± 0.04*	31.85 ± 0.6*
5 mM H_2O_2	105 ± 5.6*	1.86 ± 0.04*	0.675 ± 0.03*	0.66 ± 0.04*	29.13 ± 0.8*
5 mM H_2O_2 + 0.5 mM Cu	210 ± 10.3*	0.67 ± 0.03*	0.968 ± 0.01*	0.92 ± 0.01*	35.45 ± 0.3*
10 mM NAC	88 ± 4.9	2.5 ± 0.18	0.424 ± 0.02	0.52 ± 0.02	24.75 ± 0.3
10 mM NAC + 0.5 mM Cu	92 ± 5.3	2.28 ± 0.02	0.564 ± 0.01	0.59 ± 0.01	27.75 ± 0.3

Note: Thiobarbituric acid reactive substances (TBARS) measured employing malondialdehyde as reference standard and depicted as nmoles/min/mg protein. Glutathione levels reduced (GSH) and oxidized (GSSG) levels measured employing o-phthalaldehyde and denoted the ratio as GSH/GSSG. One unit of super oxide dismutase (SOD) represents the amount of enzyme that inhibits 50% of the rate of auto-oxidation of pyrogallol/min/mg protein. One unit of catalase activity is defined as the amount that decomposes one mmol of H_2O_2/min/mg protein. One unit of glutathione peroxidase (GPx) activity corresponds to the amount of formed GSSG in µg/min/mg protein. All values are represented as Mean ± SD. *p< 0.05 in comparison to controls as obtained from three independent experiments.

Figure 3: Influence of copper induced stress on intracellular Ca^{2+} levels. Increase in fluorescence intensity resulting from binding of crimson red to Ca^{2+} was measured in mycelia grown in various conditions. Ca^{2+} contents, expressed in nM, derived from dissociation constant (K_d = 187 nM). *p< 0.05 in comparison to controls as obtained from three independent experiments



However, combined presence of H_2O_2 and copper resulted in 38% increase of CaMK II activity. Conversely, decrease in total PKC activity (23%) was observed in under the copper induced stress conditions, but decreases (44%) in activity of Ca^{2+}-dependent PKC (Ca^{2+}-PKC) was more noteworthy ($p < 0.02$). Further, combined presence of copper and H_2O_2 in cultures caused 56% decrease in Ca^{2+}-PKC activity when compared to untreated controls. On the other hand, NAC resulted in ~33% increase of both PKC and Ca^{2+}-PKC activities under similar incubations indicated the restoration of PKC activity. However, Ca^{2+}-PKC, which normally contributes to ~47% of total PKC activity, was reduced upon transient exposure of copper and accounted for about 34%. But, pre-incubation with H_2O_2 caused marginal decrease (11%) in Ca^{2+}-PKC (Table 2).

In addition to decrease in PKC activity, TPP activity was also decreased 38 and 26% in presence of copper and H_2O_2 in cultures respectively when compared to untreated controls. In comparison to this, a decrease in the specific activity of calcineurin (52%) was more noteworthy ($p < 0.02$). Calcineurin specific activity was also decreased (33%) upon exposure to H_2O_2 alone and further decreased its activity to 60% in addition of copper. Nevertheless, an antioxidant, NAC restored the activity of TPP which was decreased due to copper induced stress with 17% increase in calcineurin activity. However, combined presence of NAC and copper in media attenuated the decrease of calcineurin activity caused by copper.

DISCUSSION

It is well documented that toxic concentrations of metals induce metallothioneins (MT) which are having antioxidant property, to prevent the oxidative damage caused by the metals. The redox metal, 0.5 mM copper induces metallothionein (CuMT) upon transient exposure of *N. crassa* to copper, but not by other stress factors unlike in higher eukaryotes (Kumar *et al*., 2005). Hence, this bread mold was chosen to study the role of protein kinases and phosphatases involved in

Table 2: Influence of Copper Induced Stress on Ca^{2+}-Dependent Protein Kinases and Phosphatases

Stress Conditions	Protein kinase C (pmoles/min/mg Protein)		CaMK II $1x10^6$dpm/min/mg Protein)	TPP (µmoles/min/mg Protein)	Calcineurin (µmoles/min/mg Protein)
	Total	Ca^{2+}-PKC			
Control	4.59 ± 0.14	2.18 ± 0.1	0.09 ± 0.009	0.17 ± 0.008	1.73 ± 0.09*
0.5 mM Cu	3.51 ± 0.02*	1.22 ± 0.05*	0.104 ± 0.007	0.082 ± 0.007*	1.07 ± 0.06*
5 mM H_2O_2	4.16 ± 0.3	1.81 ± 0.09	0.1 ± 0.004	0.114 ± 0.08*	1.28 ± 0.08*
5 mM H_2O_2 + 0.5 mM Cu	2.61 ± 0.01*	1.02 ± 0.03*	0.125 ± 0.007	0.068 ± 0.06*	1.06 ± 0.05*
10 mM NAC	6.12 ± 0.2*	2.9 ± 0.13*	0.088 ± 0.001	0.199 ± 0.03	1.82 ± 0.09
10 mM NAC + 0.5 mM Cu	4.53 ± 0.2	1.99 ± 0.12	0.094 ± 0.007	0.161 ± 0.04	1.68 ± 0.078

Note: 5 mM H_2O_2 or 10 mM N Acetyl cysteine (NAC) was included in the medium 2 h before addition of 0.5 mM copper (1 h, Cu). Ca^{2+}-dependent protein kinases and phosphatases were measured in mycelia harvested after 49 hours. One unit of protein kinase C or Ca^{2+} dependent PKC activity (Ca^{2+}-PKC) corresponds to pmoles of [γ-^{32}P]-ATP incorporated into histone III S/min/mg protein. One unit of Ca^{2+}-calmodulin dependent protein kinase II (CaMK II) activity denotes the incorporation of 1 x 10^6 dpm of [γ-^{32}P]-ATP into syntide-2/min/mg protein. One unit of total protein phosphatase (TPP) activity denotes μmole of p-nitrophenol released from the p-nitrophenyl phosphate/min/mg protein. One unit of calcineurin specific activity denotes μmole of free phosphate released from the RII peptide/min/mg calcineurin. All values are represented as Mean ± SD. *$p < 0.05$ in comparison to controls as obtained from three independent experiments.

Ca^{2+}-dependent phosphoprotein cascade on copper induced oxidative stress indicators under the conditions of CuMT induction.

Copper Induced Oxidative Stress is Concomitant with Altered Ca^{2+}-Signaling

Transient exposure of the *Neurospora* to copper caused higher metabolic rate (Figure 1) and was found to be concomitant with oxidative stress as evidenced by increased ROS accumulation (Figure 2), lipid peroxidation and unregulated the activities of antioxidant enzymes such as SOD, catalase and GPx (Table 1). Further, decreased GSH/GSSG indicated the intracellular antioxidant, GSH acting against copper toxicity. Our results are consistent with earlier reports where copper mediated toxicity increased lipid peroxidation (Mattie *et al*., 2008), depletion of GSH levels (Suntres and Lui, 2006; Speisky, 2009) and up-regulation antioxidant enzymes (Culotta *et al*., 1995; Yasokawa *et al*., 2008) in various eukaryotic systems. Recent observations made with certain diseases, cancer (Gupta and Mumper, 2009), atherosclerosis (Haidari *et al*., 2001) and cardiovascular damage (Cooper *et al*., 2009) has shown that elevated extracellular levels of copper associated with oxidative stress in human patients. Further, copper toxicity caused higher metabolic rate with increased oxygen consumption and CO_2 liberation. In addition, a prooxidant, H_2O_2 alone caused mild oxidative stress conditions and it potentiates copper induced production of ROS significantly ($p < 0.005$), lipid peroxidation ($p < 0.01$) and activities of GPx ($p < 0.01$), SOD ($p < 0.005$) and catalase ($p < 0.01$) and decrease in GSH/GSSG ($p < 0.005$) in *N Crassa* (Table 1). Such copper mediated toxicity is mitigated with an antioxidant, NAC. Recent reports equally made with NAC, attenuated copper overload oxidative damage in rat brain (Ozcelik *et al*., 2012).

Further, copper induced toxicity are known to cause changes in Ca^{2+} flux patterns (Manzl *et al*., 2004; Suntres and Lui, 2006; Gonalez *et al*., 2010; Rosenfeld *et al*., 2010) in various organisms. In present study, we have noted that copper toxicity elevated intracellular Ca^{2+} levels and further enhanced by pretreatment with H_2O_2 (Figure 3). Those levels could be mitigated by NAC ascribed to Ca^{2+} release from intracellular stores through copper mediated stress. Interestingly the cellular content of the Ca^{2+}-modulator, calmodulin and activity of CaMK II was unaffected despite the elevated intracellular Ca^{2+} levels. However, activities of PKC including that of Ca^{2+}-PKC were contrarily decreased in cultures exposed transiently to copper with similar observations made in H_2O_2 treated cultures (Table 2). These findings gain support from previous observations revealing complete inactivation of PKC under oxidative stress conditions (Chu *et al*., 2004). While TPP activity was decreased, activity of calcineurin was decreased more notably in copper mediated stress conditions without modulating the protein levels of calcineurin. Considering copper mediated oxidative stress conditions, it is to be noted that calcineurin is the only phosphatase among Ca^{2+}-signaling enzymes, which is highly vulnerable to ROS (Ullrich *et al*., 2003; Lee *et al*., 2007; Carruther *et al*., 2008) due to presence of Fe^{2+}-Zn^{2+} center in its catalytic subunit (Namgaladze *et al*., 2002). In present study, the decreased calcineurin activity and PKC could be through free radical mediated inactivation since those activities were restored with NAC treatment.

It well known that calcineurin shown a regulatory function upon its ability to

dephosphorylate transcriptional factors during stress responses with sustained elevation of CDRE response depends on intracellular Ca^{2+} and calcineurin activity (Yoshimoto *et al*., 2002; Li *et al*., 2011). CDRE plays a vital role in regulation of ethanol or salt stress adaptation through Crz1p/calcineurin-pkc/Slt2 pathways in yeast (Ruiz *et al*., 2003; Deng *et al*., 2006; Araki *et al*., 2009). Further, calcineurin play role in protection from cadmium induced testicular toxicity in mice through its inhibition (Martin *et al*., 2007). Results obtained in the present study suggest that oxidative stress induced by copper is indeed associated with significant decreases in activities of PKC and calcineurin in this lower eukaryote. Further experimentation in this regard is certain to yield important information on the gene regulatory transcription factors mediated by Ca^{2+}-signaling phosphoprotein cascade that involved in CuMT gene expression.

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