Molecular Basis of Protein Kinase C Isoforms in Oxidative Burst of Neutrophils

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Abstract: Protein kinase C (PKC) and NADPH oxidase enzyme complex play central role in the production of reactive oxygen species (ROS) during neutrophil oxidative burst. However, the examination of their roles at the molecular levels needed more clarification, especially in the presence and absence of activators and/or inhibitors. Their expression during oxidative burst was measured in Egyptian buffalo's neutrophils at both the mRNA and protein levels. From amongst 12 known PKC isoforms, only three DNA sequences which encode isoforms: PKCγ, PKCε, PKC λ were detected in the Egyptian buffalo neutrophils. The opsonized zymosan and zinc chloride stimulated the respiratory burst in concentration dependent manners which were proportional to the mRNA levels of the three PKC isoforms. Whereas, the neutrophils that incubated with salicylic acid and tricine-copper complex then stimulated with opsonized zymosan inhibited the oxidative burst and hence ROS production. However, their effects were not straightforward when measured at the molecular levels; the salicylic acid increased the transcriptional and expression levels of PKC whereas, copper complex showed an initial increase in transcriptional level then a decrase with longer incubation period. **Conclusion:** PKC isoforms afftects the rate of oxidative burst and it appeared that the dominant isoforms are dependent on the source of neutrophils. Moreover, their activities are subjected to control by the surrounding environmentals conditions and the time of exposure to simili or inhibitors.
[Noha A. M. Shendy, Mohamed M. A. El-Nagar and Yehia A. Osman Ellazeik **Molecular Basis of Protein Kinase C Isoforms in Oxidative Burst of Neutrophils]** Journal of American Science 2012; 8(4): 319-327].(ISSN: 1545-1003). http://www.americanscience.org. 43.

Key Words: protein kinase C; neutrophil; reactive oxygen species; respiratory burst; NADPH oxidase; phagocytosis.

1. Introduction

Professional phagocytic cells such as neutrophils and macrophages play a central role in defending the host against microorganisms by producing ROS via the NADPH oxidase enzyme complex.This multicomponent enzyme is dormant in unstimulated cells but can be activated by various stimuli. Activation of the NADPH oxidase is associated with phosphorylation of p47phox (phox stand for phagocyte oxidase), p67phox, the subunits of the flavocytochrome b558 and of p40phox. In the activated form, the NADPH oxidase complex mediates the transfer of electrons from cytosolic NADPH to O_2 to produce the superoxide anion (O_2^-) (Raad *et al.,* 2009). $O_2^-$is the precursor of other toxic ROS, such as hydrogen peroxide (H_2O_2), the hydroxyl radical ($HO^{\bullet}$), and hypochlorous acid (HOCl), which are involved in bacterial and other microbial destruction (El-Benna *et al.,* 2005).

A large number of soluble and particulate factors such as formylated peptides, leukotriene B4 (LTB4), platelet activating factor (PAF), opsonized bacteria, opsonized zymosan (OZ), latex particles, complement fragment C5a, diacylglycerol (DAG), and PKC activators such as phorbol-12-myristate-13-acetate (PMA) can stimulate NADPH axidase in phagocytes. Protein phosphorylation and translocation of cytosolic components to the plasma membrane are two events required for NADPH oxidase. The oxidative burst is triggered by the binding of a stimulus to a specific receptor.The receptor transmits information through the cytoplasmic membrane via a family of heterotrimeric G proteins (proteins binding GTP). Engagement of these receptors results in G protein activation with subsequent dissociation of the α from the βγ-G protein subunits and βγ-mediated activation of membrane enzymes such as phospholipase C (PLC), phospholipase A2 (PLA2), and phospholipase D (PLD). PLC catalyzes the hydrolysis of membrane phoshopholipids resulting in the formation of inositol trisphosphate (IP3) and DAG and release of arachidonic acid. IP3 induces Ca^{2+} release from intracellular stores; DAG, in conjunction with Ca^{2+}, allows for activation of PKC, which translocates from the cytosol to the plasma membrane and phosphorylates several key components of the NADPH oxidase (El-Benna *et al.,* 2005).

Inherited deficiencies in NADPH oxidase result in chronic granulomatous disease (CGD), characterized by enhanced susceptibility to microbial infection (Raad *et al.,* 2009). Therefore, it was taken as a target to examine its role in oxidative burst as an

important function of phagocytosis by neutrophils and non-specific immune response in buffalo.

2. Material and Methods

2.l. Neutrophils Isolation

Blood was collected from sacrificed adult male Buffalos, at Mansoura Slaughterhouse under supervision of resident veterinarian into ACD-containing tubes and erythrocytes removed using dextran sedimentation (6% dextran/0.9% NaCl) followed by two rounds of hypotonic lysis using ddH_2O. Neutrophils were isolated from the resulting cell suspension using Ficol-Histopaque density centrifugation. The entire isolation was done at 4°C. Purified neutrophils were suspended in phosphate buffer saline (PBS) PH 7.4, (Na_2HPO_4, KH_2PO_4, NaCl, KCl, 1.44, 0.24, 8.0, 0.2 g /L dist.H_2O, respectively) and were kept on ice until needed (Heit *et al.,* 2008). Viability of neutrophils were evaluated by Trypan blue exclusion (Strober, 2001), and the neutrophils were counted and immediately processed for further experimentation. The purity and viability of the neutrophils were >90% and >98%, respectively.

2.2 Effect of zinc chloride, zymosan, salicylic acid, tricine-copper complex, copper chloride, and tricine on respiratory burst:

Preperation of opsonized zymosan:

Opsonized zymosan was prepared as described by Kobayashi *et al.* (2009). Zymosan was suspended in Buffalo sera and incubated for 30 min at 37°C, washed twice with PBS (pH 7.4) and the washed products Were resuspended in PBS at different concentrations of 0, 1.7, 2.5, 5, 10 mg/ ml, which were used throughout this study.

2.3 Luminol chemiluminescence assay:

Luminol-derived chemiluminescence was measured as described by Rinaldi *et al.* (2007). Zymosan and zinc chloride treated neutrophil's

2.4. Chemiluminescence Assay:

Four zinc chloride concentrations (0, 200, 500, 1000 µM) were used to test their effect on the ROS production by buffalo neutrophils. For each reaction (final volume 200 µl) a 120 µL of neutrophils (2 $\times 10^5$ cells/ml) suspended in Tris-G buffer (pH 7.4) ($CaCl_2.2H_2O$, KCl, $MgSO_4$, NaCl, D-Glucose, Tris, 1.26, 5.37, 0.81, 140, 5.5, 25 mM, respectively) the proper $ZnCl_2$ conc and luminol to a final conc of 0.5mM were added and incubated at 37°C. The same protocol was used with opsonized zymosan, except the concentrations were 0, 1.7, 2.5, 5, 10 mg/ ml.

Salicylic acid, tricine-copper complex, copper chloride (metal), tricine (ligand of copper complex) treated

2.5. Neutrophils chemiluminescence assay:

Neutrophils (2 $\times 10^5$ cells/ml) were suspended in PBS, and treated with different final concentrations of salicylic acid (0,0.1,10,1000 µM) (Shapira *et al.,* 1997), copper complex (tricine-Cu^{2+}complex, prepared by Prof. Mohsen Mustafa, Chemistry Department, Masnoura Univ.) (0,20,50,200,500,1000 µM), copper chloride (0,50,100,500,1000 µM), and tricine (0,50,100,200,500,1000 µM) then incubated for 15 min at 37°C pre-chemiluminescence assay. The reaction mixture of chemiluminescence assay contained 120 µL of treated neutrophils, luminol at final concentrations of 0.5mM, 60 µL opsonized zymosan (2.5 mg/ml), and the final volume was adjusted to 200 µL with PBS.

Chemiluminescence for all previous treated samples was measured at 5 min intervals with a microplate luminometer (Monobind Inc.). Background values, defined as the mean chemiluminescence values of luminol diluted in PBS, were subtracted from all readings. A chemiluminescence index was calculated by dividing the area under the curve (AUC) of the treated neutrophils with zinc chloride, zymosan, salicylic acid, ligand (tricine), copper chloride, and copper complex by the AUC of the control multiplied by 100.

2.6. Effect of zinc chloride, zymosan, salicylic acid, tricine copper complex, copper chloride, and tricine on PKC transcription and expression in neutrophils:

2.6.1. Sample preparation:

2.6.1a. Zinc chloride and opsonized zymosan treated neutrophils:

Neutrophils (2 $\times 10^7$ cell/ml) were suspended in 100 µL of Tris-G buffer (pH 7.4) treated with zinc at final concentration of 1000 µM, kept at 37°C and sample were analyzed (RT-PCR and protein content) at different time intervals (5, 15, 30, 45 and 60min). The reactions were terminated by placing neutrophils at 0°C. Same protocol was used with opsonized zymosan, except the concentrations were 2.5 mg/ ml

2.6.1b. Salicylic acid and copper complex treated neutrophils:

Neutrophils (2 $\times 10^7$ cell/ml) were suspended in 100 µL of PBS treated with salicylic acid, or copper complex at a final concentration of (1000 µM) and incubated for 15 min. at 37^0C, then the cells were stimulated with opsonized zymosan (2.5 mg/ml) at different time point (5-60min) at 37°C, the

stimulation of neutrophil was terminated by placing neutrophil at 0°C.

2.6.1c. Polyacrylamide gel electrophoresis of neutrophil proteins:

To examine the level of PKC isoforms in treated and untreated neutrophils, the total cellular protein of neutrophils were separated according to their molecular weights by Sodium dedocylsulfate polyacrylamide gel electrophorosis (SDS-PAGE) (Laemmli, 1970).

2.6.1d. Nucleic acid Extraction:

Total nucleic acids were isolated as following. The suspended cell pellet of each sample was mixed with 300 µL of lysis solution (37.5mM Na–citrate, pH 7.0, 6M guanidine thiocyanate (GTC); 0.75% Sarcosyl NL30; 2% 2-mercaptoethanol). After 5min incubation on ice, the lysate was mixed with 900 µL of cold isopropanol. After additional 30min incubation on ice, the formed precipitate was pelleted. The pellet was redissolved in 400 µL of a 1.5-fold diluted lysis solution , reprecipitated with 800 µL of isopropanol. The pellet was washed by 400 µL of an ice-cold alcohol:saline solution (a 45:55 v/v mixture of ethanol with 200mM Na–citrate, 300mM NaCl, and 0.4mM EDTA) and dissolved in 500 µL of 10mM Tris–HCl (pH 8.0), 8M urea, 1mM EDTA, 5% SDS, and 2% 2-mercaptoethanol and shaked for 10min. Then 40 µL of 4M NaCl was added to the sample before being extracted by 540 µL of phenol:chloroform:isoamyl alcohol (25:24:1,v/v/v). A 400 µL portion of the aqueous phase was again extracted with an equal volume of chloroform, and then precipitated with two volumes of absolute ethanol. The nucleic acid pellet was washed with 70% ethanol, dried and dissolved in 20 µL of water and nucleic acid samples were then treated with deoxyribonuclease (DNase) made by Fermentas company to remove genomic DNA and stored at -70°C until used. The integrity and quality of RNA were determined by 2% (w/v) agarose gel electrophoresis, while the concentration was determined spectrophotometrically (GENWAY 6305 spectrophotometer) where one O.D260 unit approximately is equal to 40µg of RNA/ml (Chetverina *et al.,* 2004).

2.6.2. Reverse Transcription-Polymerase Chain Reaction

Levels of mRNA expression were measured using semi quantitative RT-PCR (RT-PCR kit made by Fermentas company), in which the DNase-treated RNA was used to synthesize cDNA. The cDNA was used again with the primers published by Zorilla *et al*., 2009 (Table 1). The PCR was performed as follow: the template cDNA was initially denatured at 94°C for 3 min, followed by 28 cycles for amplification of the housekeeping gene (H2A) and 33 cycles for (all PRKC isoforms. Each of the subsequent PCR cycle consisted of denaturation at 94°C for 30s; annealing at 50°C for1min and extension at 72°C for1min. At the of the programmed cycles an extra extention step at 72°C for 5min was performed and the PCR products were separated by gel electrophoresis, photographed and analyzed.

3.Results

3.1. Cell Viability and oxidative burst assay

The viability of neutrophils were not affect at all by tricine and salicylic acid while $CuCl_2$ and Cu-complex reduced their viability by 26% and 12% respectively at the used concentration of 1000 µM.

Repiratory burst was measured as ROS production as a function of opsonized zymosan concentration (Figure 1A) ROS production increased as zymosan conc increase and reached steady-state at concentration from 2.5 until 5 mg/ml and a steady decline was observed at higher concentrations, i.e. higher concentrations than 5 mg/ml have an inhibitory effect on ROS production. The chemiluminescence was quantified by area under curve of the chemiluminescence dose-response curve and the chemiluminescence index was calculated to be 193, 256, 263, 177%, at zymosan concentrations of 1.7, 2.5, 5, 10 mg/ml, respectively.

Zinc chloride increased chemiluminescence index by 7, 30.5, 393% at concentrations of 200, 500, 1000 µM, respectively. The current results showed that the effect of zinc chloride on neutrophils oxidative burst was a concentration -dependent which is reflected of increased ROS production (Figure 1B). In contrast the salicylic acid has an inhibitory effect on neutrophils ROS production in a concentration dependant manner. As salicylic acid decreased chemiluminescence index by 17%, 31%, and74% at concentrations of 0.1, 10, and 1000 mM, respectively (Figure 1C). The ROS production of tricine (ligand) treated neutrophils was decreased by 33%, 44%, and 48% under the effect of the corresponding concentration of added tricine at 50, 100, and 200 µM, respectively, then gradual increase was significantly observed at concentration over 500 µM (Figure 1D).

The inhibitory effect of the copper complex on ROS production, by OZ stimulated Buffalo neutrophils was a concentration-dependent process (Figure 1E). The chemiluminescence indexes showed a sharp decrease at 20 -50 µM of the complex that reached 27, 81% , respectively. Higher concentrations such as 200 µM showed almost complete inhibition whiched reached 93%,

concentrations more than 200 µM produced 100% inhibition of oxidative burst.

Figure 1(F) showed that copper chloride has stimulatory effect at lower concentrations as chemiluminescence index increased by 18%, 137%, and 126% at concentrations of 50, 100, and 200 µM respectively. While at higher concentations of 500 and 1000 µM the chemiluminescence index decreased by 14% and 66% , respectively.

Table 1: Primer sequences used for polymerase chain reaction.

Gene	Product size	Sequences	Cycles
PRKCA	356	*F* 5´-AATCGTTTCGCCCGCAAAG-3´ *R* 5´- TCACAGGTGTCACATTTCATCCC-3´	33
PRKCB1	256	*F* 5´-TGCTGTATGAAATGTTGGCTGG-3´ *R* 5´--CGTGAATCTCTTTGCGTTGC-3´	33
PRKCB2	254	*F* 5´-CTGTATGAAATGTTGGCTGGGC-3´ *R* 5´- GGCTGAATCTCTTTGCGTTCG-3´	33
PRKCG	101	*F* 5´- AAAGGCAGTTTTGGGAAGG-3´ *R* 5´–CGTCATCCTGGACAATCACG-3´	33
PRKCD	391	*F* 5´-ACATCAAGATTGCCGACTTTGG-3´ *R* 5´-AACCTCCGCTTTTCCAGCAG-3´	33
PRKCE	227	*F* 5´-TTTTGGAACGAGCAGAATGAGG-3´ *R* 5´-GCCACAGAGAACCCTTGGAAATC-3´	33
PRKCH	453	*F* 5´-GGGGATAATGCGACAAGGACTTC-3´ *R* 5´-TGGGTGAGGAAAGGGTGATTGC-3´	33
PRKCQ	470	*F* 5´-TCACTCAGTTCTTCTGCTGCTTTC-3´ *R* 5´-GACCACCTCATCATTCAGTATGGC-3´	33
PRKCI	408	*F* 5´-GCAAATCAAAATCCTGAAGCCC-3´ *R* 5´-ATAAGTCCATCTACCGCCGAGGTG-3´	33
PRKCL	222	*F* 5´-ACAAGGACCCAAAGGAACGC-3´ *R* 5´-TCACAATGTCATCGTCATCTGGAG-3´	33
PRKCZ	491	*F* 5´-TGCCAGATTCTACGCTGCTGAG-3´ *R* 5´-CGTGAGACTTGATGTCGGAAAACC-3´	33
PRKD1	206	*F* 5´-TGTGACTTTGGTTTTGCCCG-3´ *R* 5´-TGGATTTGGTCGTGGATGTCC-3´	33
H2A	209	*F* 5´-AGGACGACTAGCCATGGACGTGTG-3´ *R* 5´-CCACCACCAGCAATTGTAGCCTTG-3´	28

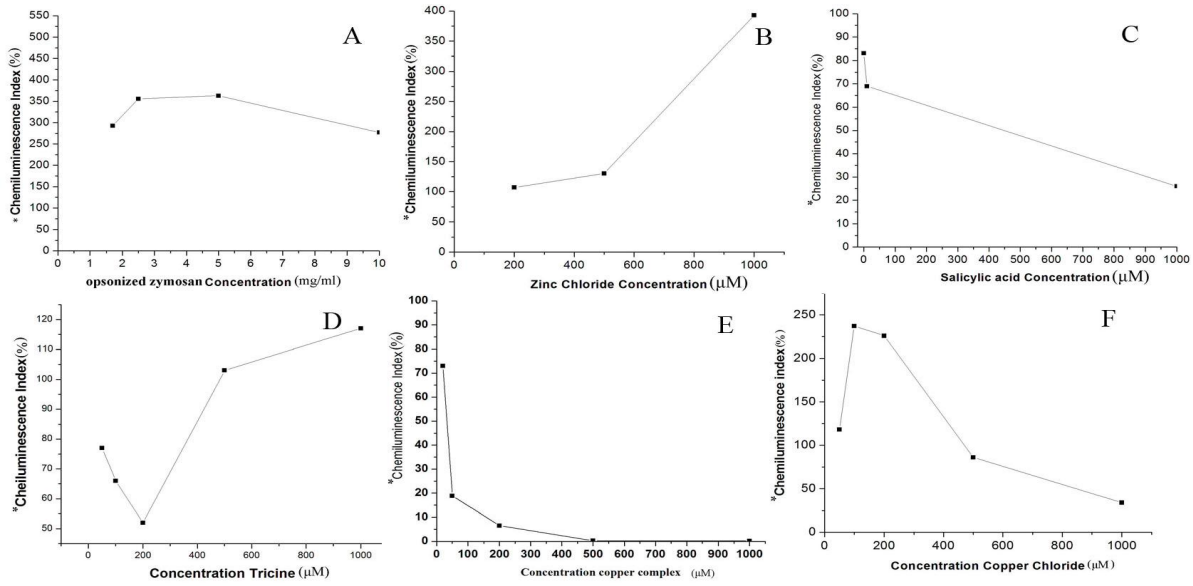

Figure 1: Influence of different concentrations of (A) opsonized zymosan (1.7, 2.5,5,10 mg/ml), (B) zinc chloride (200, 500, 1000 µM), (C) salicylic acid (0.1, 10, 1000 µM), (D) tricine (50, 100, 200, 500, 1000 µM), (E) copper complex (20, 50, 200, 500, 1000 µM), (F) copper chloride (50, 100, 200, 500, 1000 µM) on chemiluminescence index of Buffalo neutrophils.*Chemiluminescence index = (AUC of the treated cells/ AUC of the control) x100.

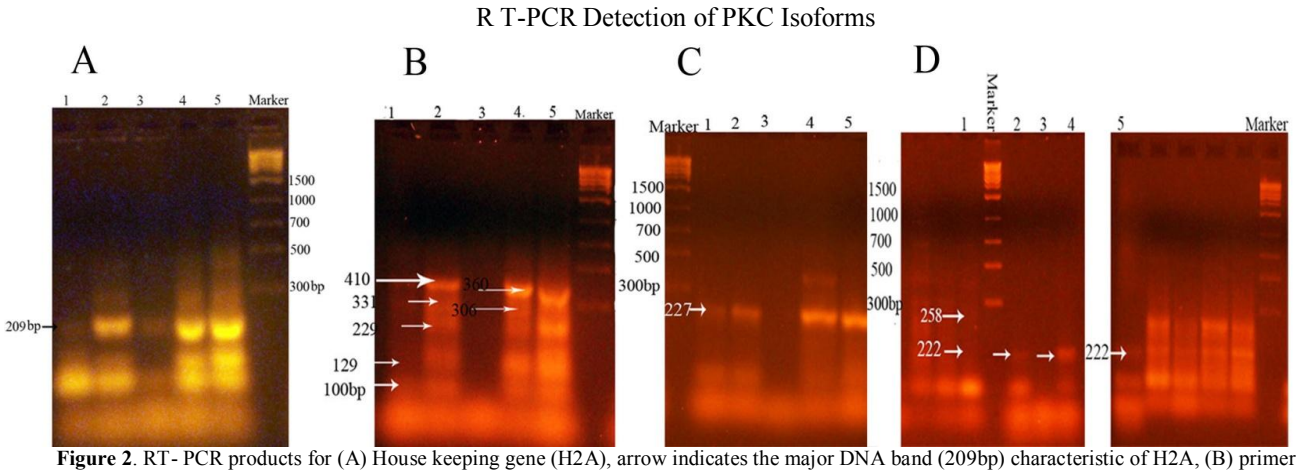

Figure 2. RT- PCR products for (A) House keeping gene (H2A), arrow indicates the major DNA band (209bp) characteristic of H2A, (B) primer PKCγ arrow indicates the DNA band (100bp) characteristic of PKCγ, (C) primer PKCε arrow indicates the DNA band (227bp) characteristic of PKCε, (D) primer PKCλ arrows indicate the DNA band (222bp). Lane 1: control sample, lane 2: $ZnCl_2$ - treated sample (45 min), lane 3: zymosan-treated sample (15 min), lane 4: salicylic acid-treated sample (60 min), lane 5:Cu-complex -treated samples (5 min).

Only the housekeeping gene and three PKC isoforms (PKCγ, PKCε,PKCλ) gave typical PCR products, i.e. DNA bands of about 209, 101, 227, 222 bp, respectively. The specific primers to all other PKC isoforms produced negative PCR profiles even with the control sample. The housekeeping gene isoform specific primer did not produce the typical DNA band with the sample treated with a zinc chloride for 5min and the zymosan treated sample for 50 min. The primer specific for the gene encoding for isoform PKCγ did not produce the typical DNA band with the zymosan-treated samples (5, 15 min). The primer specific for the isoform PKCε gene produced negative results with the samples treated with zinc chloride (5, 30 min), zymosan (15,50 min.), salicylic acid for 50 min, and sapmles treated with Cu-complex (15, 30min). The primer specific for the isoform PKCλ produced the typical DNA band with control and sample traeated with copper complex only (45 min.). The isoforms which did not produce the typical PCR product profile with the control sample are assumed to be absent from the neutrophile under study.

3.2. Quantitative Analysis of the PCR Product:

Measurment of PCR product indicated that, zinc chloride, zymosan, salicylic acid, and copper complex increased cDNA levels of the PKC isoforms γ,ε, and λ. The increase for PKCγ by the different treatments are 30, 17% after 5, 45min of zinc chloride exposure, 30% after 45min of exposure to zymosan, 50% and 73% after 15 and 50 min exposure to salicylic acid, while copper complex increased gene expression by 34% after 5 min of exposure then the transcriptional level decresed to 14% with longer exposure . The cDNA level of isoform PKCε increased 78% at the end of 45 min exposure to zymosan. Salicylic acid and copper complex increased cDNA level by 78% and 55% after 5 min, respectively, while, longer exposure down regulated its level to 44% and 25% increase after 1 h and 45 min, respectively. Zinc Chloride effects on the gene transcriptional levels was low, 14% after 45 min of exposure . Zinc chloride increased increased the gene expression of PKCλ by 42% after 30 min, zymosan increased the expression by 27, 37% after 45, and 50 min, respectively, and salicylic acid increased the expression to 85, 40, 35, 18% after 5,15,45,50 min, respectively. On the other hand, copper complex did not increase its expression over control level significantly.

3.3. Protein Electrophoresis of Neutrophils Protein Content Result

The salicylic acid, zymosan, copper complex, and zinc chloride treatment induced the expression of two PKC isoforms with molecular weights ~74 kDa and ~67 kDa. These proteins coincide with those of PKC isoforms gamma and lambda. Whereas,salicylic acid treatment induce the expression of an additional protein band (~89 kDa) corresponding to the PKC epsilon isoform.

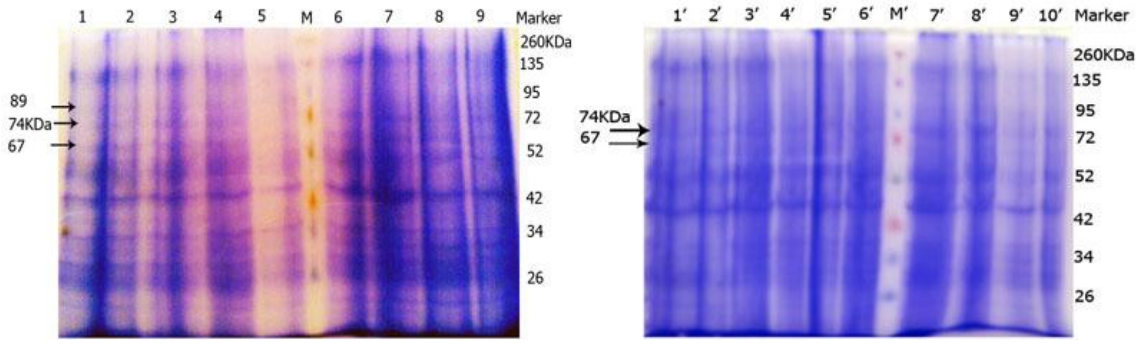

Figure 3 SDS–PAGE protein profiles of : lane 1,2,3,4 protein extracted from salicylic acid- treated neutrophils (incubation period (5,15,45,50,60 min.), lane 5 protein extracted from control,lane 6,7,8,9 protein extracted from zinc chloride- treated neutrophils (5,15,45,50,60 min.), lane 1′,2′,3′,4′ protein extracted from zymosan- treated neutrophils (incubation period extracted from Cu-complex- treated neutrophils (5,15,45,50,60 min)

4. Discussion

The role of PKC isoforms in respiratory burst (ROS) was reemphasized in the presence of some activator and/or inhibitors such as OZ, Zn ions, salicylic acid, and a Cu-Tricine complex. The OZ up to 2.5 mg/ml, zinc chloride up to 1000 µM and lower concentrations of Cu^{+2} increased ROS production. consistent with the genral trends in the literature. While, salicylic acid inhibited the respiratory burst of OZ stimulated neutrophils in a concentration dependent manner, as in several published reports (Sagone and Husney, 1987; Takeyama *et al.,* 1995; Shapira *et al.,* 1997 Benoni *et al.,* 1998; Lindahl *et al.,* 1998; Freitas *et al.,* 2010; Jomova and Valko, 2011). However, a locally prepared tricine-Cu^{2+} complex, an extremly potent inhibitor, inhibited ROS production by buffalo neutrophils completely.

The activation or inhibition of the ROS production was evident from the expression levels of PKC isozymes (PKCγ, PKCε, PKC λ) at the mRNA and protein levels. Not only DNA sequences for these three isozymes were detected in the Egyptian buffalo neutrophils, but also three protein bands with apparent molecular weights of about 82, 74, 69 KDa corresponding to these protein kinase C isoforms PKCε, PKCγ and PKC λ were detected as well in the SDS-PAGE analysis. OZ and Zinc increased the transcriptional level of mRNA and the proteins of PKCγ and PKC λ consistent with the ROS production. Our data suggests that the presence of the complete genes encode for these protein kinase C isoforms in the buffalo neutrophils, not partially conserved sequence in the genome which can be detected by the PCR technology.

So the molecular basis of ROS production emphasized the role of PKC in the zymosan and zinc induction and activaion of NADPH oxidase in buffalo neutrophils as evident from the PCR detection of their genes and the expression levels of these isozymes.

In our experimental settings, the decline of ROS production at higher concentration of zymosan was probably due the death of neutrophils leading to cessation of oxidative burst or disappearance of opsonized zymosan by unknown mechanism. However, Easmon *et al.* (1980) attributed the decline of ROS production to light quenching during chemiluminescence assay rather than a decline in metabolic phagocytic activity. Recently, the role of OZ in oxidative burst was mediated through specific membrane binding process (Nagahata *et al.,* 1993; Xia *et al.,* 1999; Kobayashi *et al.,* 2009). They demonstrated the role of a complement proteins such as C5, which reacts with a specific receptor (CD88) on neutrophils leading to stimulation PKC and hence involvement in ROS production. Moreover, Mobberley-Schuman and Weiss(2005) have reported that the zymosan opsonized with C3bi complement protein binds to CR3 receptor, which regulates signaling pathways involved in gene expression and cytoskeletal rearrangements and ultimately influences cell adherence, migration, and generation of the oxidative burst. However, a direct activation of PKC, bypassing signaling through membrane-receptor, via neutrophil stimulation with PMA is known to stimulate NADPH oxidase and hence ROS production (Kobayashi *et al.,* 2009).

The reports about the stimulatory effects of zinc was widely accepted in the literature (Takeyama *et al.,* 1995; Benoni *et al.,* 1998; Lindahl *et al.,* 1998; Freitas *et al.,* 2010). Freitas *et al.*(2010) reported the stimulatory effect of the lower zinc (5-12.5 µM) concentrations in the production of superoxide anion via activating NADPH oxidase. While, higher concentrations (up to 1000 µM) of zinc result in a rapid conversion of O_2^- to O_2 and H_2O_2 either by superoxide dismutase or spontaneously, which in turn

is used by myleoperoxidase (MPO) to generate HOCl. However, (Henderson *et al.,* 1988; DeCoursey *et al.,* 2003) had published inhibitory effect of zinc on the oxidative burst especifically on O_2^- production. Overall, the positive effects of Zn ion on oxidative burst was evident in our experiments consistent with the mainstream of the literature.

A Tricine-Cu^{2+} complex inhibited completely the ROS production, where lower concentrations of Cu^{+2} increased ROS production, probaply due to its redox activity or redox cycling reactions which led to production of reactive radicals such as superoxide anion radical and nitric oxide in biological systems (Jomova and Valko, 2011). The inhibition in ROS production at higher concentration of copper may be due to its toxicity, for example, copper at concentration of 1000 µM reduced the cell viability to 73% and also copper is also capable of causing DNA strand breaks and oxidation of bases via ROS and that lead to reduction in cell viability and its ability to produce more reactive oxygen species (Jomova and Valko, 2011).

In case of copper-tricine complex, the inhibition of ROS was not associated with cell death, as indicated by trypan blue dye, but may be attributed to their marked superoxide-dismutase (SOD) mimetic activity which inhibited ROS production (Iakovidis *et al.,* 2011). Since the dismutation reaction involves the redox cycle of Cu (II) and Cu (I), it is reasonable to expect that the redox potential of Cu (II) complex/Cu (I) complex can influence the SOD-like activity, while the ligand of the complex determines the redox potential (Kimura *et al.,* 1981).

Salicylic acid was reported to possess several inhibitory mechanisms for the oxidative burst and ROS production by neutrophils stimulated with OZ (Sagone and Husney, 1987; Shapira *et al.,* 1997). Salicylic acid and/or aspirin (ASA) were decarboxylated by the hydroxyl free radical generated by the enzyme system xanthine-xanthine oxidase which made act as scavengers for ROS. Moreover, they have established that the hydroxyl free radical (OH^-) produced by stimulated granulocytes did decarboxylate and hydroxylate benzoic acid, the parent compound of the salicylates, and this might relate directly to their known anti-inflammatory properties. Moreover, Yan *et al.* (2005) reported that salicylic acid is commonly used as trapping agent, has a relatively high sensitivity for OH^-. Hydroxylation of salicylic acid produces two isomers, 2, 3- and 2, 5-dihydroxbenzonic acid (DHBA) and also inhibits cyclooxygenase activity, which is itself an important source of ROS.

Again, our results are in general agreement with other published reports by several investigators, where the zymosan induced translocation of PKC from the cytosol to membranes in intact cells and triggered NADPH oxidase activation and oxidative burst (Duyster *et al.,* 1992; Sergeant and McPhail, 1997; Yamamori *et al.,* 2000; Fontayne *et al.,* 2002). They further explained that once NADPH oxidase was activated, p47phox becomes phosphorylated on several sites and translocated to the plasma membrane where it interacts with cytochrome b_{558} which believed to be mediated by the PKC family. On the other hand, Allard (1996) showed that PKC is not involved in the OZ activation of neutrophils and phosphorylation may be accomplished by protein-tyrosine kinases or protein-serine kinases. However, the activation of NADPH oxidase by zinc via PKC, led to the formation of superoxide radical (O_2^-). Where zinc activates PKC by forming a zinc finger with the enzyme. Accepting that, all PKC isoforms have four conserved domains, C1–C4, from which the C1 domain contains cysteine rich finger-like motifs, and this binds to two zinc atoms forming a zinc finger motifs. These modulate diacylglycerol binding increasing the intracellular zinc concentrations that could influence the activity and localization of PKC family members (Chou *et al.,* 2004). Similar to OZ and zinc, salicylic acid did increase the transcriptional levels (mRNA and their proteins) of PKCγ, PKCε and PKC λ contradicting the biochemical measurements of the oxidative burst. Where, the salicylic acid was reported to inhibit the respiratory burst by scavenging ROS, not the PKC transcription or expression levels, as shown by RT-PCR and protein electrophoresis patterns. These data are in agreement with those reported by Eastwood (2001), where they repoted that unconjugated salicylic acid increased gene expression in both plants and mammalian cell line system and may be due to decreased of proteolytic activity as reported by Kirillova *et al.* (2011).

The apparent contradiction of the locally prepared tricine-copper complex that resulted in increasing the transcriptional level (the amount of mRNA) of PKCγ, PKCε and PKC λ at the begining and at longer incubation it significantly decreased their expression. This is may be attributed to its action as chemical nucleases and degradation of the DNA itself (Haribabu and Reddy, 2011). It is also apparent that zymosan could have protected DNA from degradation and/or resatbilized mRNA leading to increases in PKC level. In conclusion, our data resulted in the discovery of lower number of PKC isoforms in Egyptian buffalo than what is widely reported in the literature. Also, the locally prepared Tricine-copper complex proved to be an extremely potent inhibitor at all concentrations used, depsite the known stimulatory effect of Cu ions at lower

concentration for the oxidative burst. Further experiments have to be done to proof this hypothesis.

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References

Allard, B. (1996): Dependence of superoxide anion production on ectracellular and intracellular calcium and protein kinase C in bovine neutrophill.Department of Animal Science.Montréal Macdonald Campus of McGill University.Master..

Benoni, G., L. Cuzzolin, M. Marrella, F. Crivellente, A. Adami, C. Bertaso, G. Velo and R. Milanino(1998): Neutrophil behavior following exposure to in vivo or in vitro zinc in normal and acutely-inflamed rats: studies on lysozyme secretion, superoxide anion release and platelet adhesion. *Inflammation*; 22(2): 175-189.

Chetverina, H. V., M. V. Falaleeva and A. B. Chetverin(2004):Simultaneous assay of DNA and RNA targets in the whole blood using novel isolation procedure and molecular colony amplification. *Anal Biochem*; 334(2): 376-381.

Chou, S. S., M. S. Clegg, T. Y. Momma, B. J. Niles, J. Y. Duffy, G. P. Daston and C. L. Keen(2004): Alterations in protein kinase C activity and processing during zinc-deficiency-induced cell death. *Biochem. J.*; 383(1): 63-71.

DeCoursey, T. E., D. Morgan and V. V. Cherny(2003): The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. *Nature*; 422(6931): 531-534.

Duyster, J., A. Schulze-Specking, E. Fitzke and P. Dieter(1992): Protein kinase C involved in zymosan-induced release of arachidonic acid and superoxide but not in calcium ionophore-elicited arachidonic acid release or formation of prostaglandin E2 from added arachidonate. *J Cell Biochem*; 48(3): 288-295.

Easmon, C. S., P. J. Cole, A. J. Williams and M. Hastings(1980): The measurement of opsonic and phagocytic function by Luminol-dependent chemiluminescence. *Immunology*; 41(1): 67-74.

Eastwood, M. A. (2001): A molecular biological basis for the nutritional and pharmacological benefits of dietary plants. *QJM*; 94(1): 45-48.

El-Benna, J., P. M. Dang, M. A. Gougerot-Pocidalo and C. Elbim(2005): Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp (Warsz)*; 53(3): 199-206.

Fontayne, A., P. M. Dang, M. A. Gougerot-Pocidalo and J. El-Benna (2002): Phosphorylation of p47phox sites by PKC alpha, beta II, delta, and zeta: effect on binding to p22phox and on NADPH oxidase activation. *Biochemistry*; 41(24): 7743-7750.

Freitas, M., G. Porto, J. L. Lima and E. Fernandes(2010): Zinc activates neutrophils' oxidative burst. *Biometals*; 23(1): 31-41.

Haribabu, P. and K. H. Reddy(2011): DNA binding and cleavage activities of cationic dinuclear copper(II) and nickel(II) complexes with novel oxime-thiosemicarbazones. *Indian Journal of Chemistry*; 50A: 996-1001.

Heit, B., L. Liu, P. Colarusso, K. D. Puri and P. Kubes(2008):PI3K accelerates, but is not required for, neutrophil chemotaxis to fMLP. *Journal of Cell Science*; 121(2): 205-214.

Henderson, L. M., J. B. Chappell and O. T. Jones(1988): Superoxide generation by the electrogenic NADPH oxidase of human neutrophils is limited by the movement of a compensating charge. *Biochem J*; 255(1): 285-290.

Iakovidis, I., I. Delimaris and S. M. Piperakis(2011): Copper and its complexes in medicine: a biochemical approach. *Mol Biol Int* .,2011: 1-13.

Jomova, K. and M. Valko(2011): Advances in metal-induced oxidative stress and human disease. *Toxicology*; 283(2-3): 65-87.

Kimura, E., A. Sakonaka and M. Nakamoto(1981): Superoxide dismutase activity of macrocyclic polyamine complexes. *Biochimica et Biophysica Acta (BBA) - General Subjects*; 678(2): 172-179.

Kirillova, N. V., V. Belykh Iu and A. I. Cpasenkov (2011): Influence of salicylic acid on biosynthesis of nucleic acids in Polyscias filicifolia cell culture under the action of unfavorable temperatures. *Prikl Biokhim Mikrobiol.*; 47(4): 474-478.

Kobayashi, S., R. Sato, Y. Abe, O. Inanami, H. Yasui, K. Omoe, J. Yasuda, C. Hankanga, S. Oda and J. Sasaki (2009): Canine neutrophil dysfunction caused by downregulation of β2-integrin expression without mutation. *Veterinary Immunology and Immunopathology*; 130(3-4): 187-196.

Laemmli, U. K.(1970): Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*; 227(5259): 680-685.

Lindahl, M., P. Leanderson and C. Tagesson(1998): Novel aspect on metal fume fever: zinc stimulates oxygen radical formation in human neutrophils. *Hum Exp Toxicol*; 17(2): 105-110.

Mobberley-Schuman, P. S. and A. A. Weiss(2005): Influence of CR3 (CD11b/CD18) expression on

phagocytosis of Bordetella pertussis by human neutrophils. *Infect Immun*; 73(11): 7317-7323.

Nagahata, H., H. Nochi, K. Tamoto, H. Taniyama, H. Noda, M. Morita, M. Kanamaki and G. J. Kociba (1993): Bovine leukocyte adhesion deficiency in Holstein cattle. *Can J Vet Res.*; 57(4): 255-261.

Raad, H., M. H. Paclet, T. Boussetta, Y. Kroviarski, F. Morel, M. T. Quinn, M. A. Gougerot-Pocidalo, P. M. Dang and J. El-Benna (2009): Regulation of the phagocyte NADPH oxidase activity: phosphorylation of gp91phox/NOX2 by protein kinase C enhances its diaphorase activity and binding to Rac2, p67phox, and p47phox. *FASEB J*; 23(4): 1011-1022.

Rinaldi, M., P. Moroni, M. J. Paape and D. D. Bannerman (2007): Evaluation of assays for the measurement of bovine neutrophil reactive oxygen species. *Veterinary Immunology and Immunopathology*; 115(1–2): 107-125.

Sagone, A. L., Jr. and R. M. Husney(1987): Oxidation of salicylates by stimulated granulocytes: evidence that these drugs act as free radical scavengers in biological systems. *J Immunol.*; 138(7): 2177-2183.

Sergeant, S. and L. C. McPhail. (1997): Opsonized zymosan stimulates the redistribution of protein kinase C isoforms in human neutrophils. *J Immunol.*; 159(6): 2877-2885.

Shapira, L., Y. Schatzker, I. Gedalia, R. Borinski and M. N. Sela(1997): Effect of amine and stannous fluoride on human neutrophil functions in vitro. *J Dent Res.*; 76(7): 1381-1386.

Strober, W. (2001): Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology, John Wiley & Sons, Inc..

Takeyama, Y., K. Ogino, H. Segawa, H. Kobayashi, T. Uda and T. (1995): Houbara.Effects of zinc on production of active oxygen species by rat neutrophils. *Pharmacol Toxicol.*; 76(1): 50-55.

Xia, Y., V. Vetvicka, J. Yan, M. Hanikyrova, T. Mayadas and G. D. Ross(1999):The beta-glucan-binding lectin site of mouse CR3 (CD11b/CD18) and its function in generating a primed state of the receptor that mediates cytotoxic activation in response to iC3b-opsonized target cells. *J Immunol.*; 162(4): 2281-2290.

Yamamori, T., O. Inanami, H. Nagahata, Y. Cui and M. Kuwabara(2000): Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. *FEBS Lett* .; 467(2-3): 253-258.

Yan, E. B., J. K. Unthank, M. Castillo-Melendez, S. L. Miller, S. J. Langford and D. W. Walker. (2005): Novel method for in vivo hydroxyl radical measurement by microdialysis in fetal sheep brain in utero. *J Appl Physiol.*; 98(6): 2304-2310.

Zorrilla, L. M., M. S. Irvin and J. E. Gadsby(2009): Protein kinase C isoforms in the porcine corpus luteum: temporal and spatial expression patterns. *Domest Anim Endocrinol.*; 36(4): 173-185.