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Insulin Induced Alteration in Post-Translational Modifications of Histone H3 under Hyperglycemic Condition in L6 Skeletal Muscle Myoblasts

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Running Title: Hyperglycemia alters insulin induced histone modifications

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Summary

Chromatin remodelling events, especially histone modifications are proposed to form the mainstay for most of the biological processes. However, the role of these histone modifications in the progression of diabetes is still unknown. Hyperglycemia plays a major role in diabetes and its complications. Present study was undertaken to check the effect of insulin on alterations in post-translational modifications of histone H3 in L6 myoblasts under hyperglycemic condition. We provide first evidence that insulin under hyperglycemic condition alters multiple histone modifications by enhanced production of reactive oxygen species. Insulin induces dose dependant changes in Lysine 4 and 9 methylation, Ser 10 phosphorylation and acetylation of histone H3. Interestingly, insulin induced generation of reactive oxygen species induces dephosphorylation and deacetylation of histone H3. Preincubation with catalase and DPI prevents these changes in posttranslational modifications of histone H3. Furthermore, changes in histone H3 phosphorylation was found to be independent of ERK, p38, RSK2 and MSK1. Moreover, serine/threonine phosphatase inhibitor, okadaic acid attenuates insulin induced dephosphorylation and deacetylation of histone H3, suggesting role of serine/threonine phosphatases in altering modifications of histone H3. These changes in epigenetic modifications can provide new insights into pathogenesis of diabetes.

Key word: Insulin, hyperglycemia, L6 skeletal muscle, histone modification, oxidative stress, and Serine/threonine phosphatase

Abbreviations: Cat, Catalase; CM-DCF-DA, 5, 6-chloromethyl-2',7'-dichlorofluorescein diacetate; DPI, diphenylene iodonium; ERK, extracellular regulated kinase; HAT, histone

acetyltransferase; HDAC, histone deacetylase; MAPK, mitogen activated protein kinase; NOX, NADPH oxidase; MSK1, mitogen activated stress kinase; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; ROS, reactive oxygen species; RSK2, 90 kDa ribosomal S6 kinase.

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Introduction

Despite of intensive research efforts focused on involvement of oxidative stress in pathophysiology of diabetes and its associated complications, the role of cellular adaptive responses to oxidative stress in developing insulin resistance still remains incompletely understood. Several reports have shown that, hyperglycemia associated with diabetes generates free radicals which ultimately alters insulin signaling and thus can lead to insulin resistance [1]. Recently, it has been reported that acute fluctuations in levels of glucose produces strong deleterious effects rather than sustained hyperglycemia in diabetic patients [2]. Growing body of evidence suggests that H_2O_2 is a ubiquitous intracellular messenger [3-5]. Insulin also generates transient burst of H_2O_2 in various insulin responsive tissues such as adipocytes, hepatocytes and neuronal cells, which results in distal modifications of insulin post receptor signaling [3-5]. Ironically, insulin under high glucose condition enhances H_2O_2 generation through membrane bound NADPH oxidase (NOX) [6]. It has also been shown that depending upon concentration and duration of oxidative stress, distal insulin signals can decrease or increase [6-8]. Furthermore, several reports have shown oxidative stress can modulate insulin induced mitogen activated protein kinases (MAPKs), which in turn alters insulin induced gene expression [9, 10].

Chromatin modifications are the penultimate event leading to changes in gene expression. Many biological pathways results in covalent modifications of histones by acetylation, phosphorylation and methylation, leading to chromatin remodeling and gene regulation [11]. MAPKs such as p38 and ERK plays an important role in regulating gene expression [12-14]. Activation of these kinases activates series of downstream kinases such as RSK2 and MSK1 which results in phosphorylation of histone H3 [15, 16]. Serine/threonine phosphatases such as PP1 and PP2A are shown to be involved in dephosphorylation of histone H3 [17, 18].

Accumulating evidences also suggests coordinated changes in acetylation of histones in concurrence with the histone phosphorylation leading to the activation of genes [19, 20]. These coordinated processes are regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) enzymes in tandem with histone kinases and phosphatases [21, 22]. Recent studies have also shown that reactive oxygen species (ROS) can also alter transcription of genes by modifying histone acetylation and phosphorylation levels [23, 24].

Transcriptional regulation of genes involves multiple histone modifications. Changes in transcription can be elicited by inhibition or activation of one of several different histone modifications pathways [11]. Phosphorylation at Ser10 and acetylation of H3 are known to be involved in gene activation [20, 23]. Likewise, methylation of histone H3 at Lysine 4 is associated with gene activation and that of Lysine 9 with gene inactivation [25, 26]. Although, these reports suggest role of methylation, acetylation and phosphorylation of histone H3 in transcriptional regulation, but precise status of these modifications with respect to changes in insulin induced gene expression, associated with diabetes is still not clear.

Capitalizing on the earlier reports, the role of oxidative stress in altering histone modifications and considering the inherent lacunae in literature regarding the role of epigenetic changes in diabetes associated with insulin resistance, present study was designed to check the effect of hyperglycemia on insulin induced oxidative stress and alterations in posttranslational modifications of histone H3.

Materials and Methods

Chemicals: L6 skeletal muscle cell line was purchased from American Type Cell Culture (ATCC). Cell culture media, antibiotic solution, fetal calf serum, and trypsin–EDTA solution were purchased from GIBCO (U.S.A). Insulin was purchased from Novartis Pharma, H₂O₂ and okadaic acid from Calbiochem USA, CM-DCF-DA from Molecular Probes (Invitrogen U.S.A.), MTT, catalase and diphenylene iodonium (DPI) from Sigma Chemical Co. (St. Louis, MO, U.S.A). ECL detection kit and ECL hyperfilm were obtained from Amersham Bioscience (U.S.A). Anti-p38 antibodies was purchased from Santa Cruz biotechnology (Santa Cruz, CA, U.S.A), Anti acetylated H3, Anti monomethyl histone H3-K4, Anti-monomethyl-histone H3-K9 and Anti histone H3 were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). p-MSK 1 (Thr 581) and p-p90 RSK2 (Thr 359/ Ser 363) were purchased from Cell signaling technology (U.S.A.), p-p38 (Tyr 180/Tyr 182) was purchased from Calbiochem (U.S.A.), Anti-Actin from Sigma and Horseradish peroxidase (HRP)-conjugated secondary antibody from Santa Cruz biotechnology (Santa Cruz, CA, U.S.A). All the other chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise mentioned.

Cell Culture: L6 skeletal muscle cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (Penicillin 100 IU/ ml and Streptomycin 100 mg/ml) in 5% CO₂ at 37°C. At 80-90% confluence, L6 myoblasts were treated with 5 mM Glucose + 20 mM mannitol in case of normal glucose (or low glucose) and 25 mM glucose in case of high glucose for 5 hr and stimulated with insulin (100 nM) for the indicated time interval. In case of catalase (H₂O₂ scavenger, 0.008% w/v) or DPI (NADPH oxidase inhibitor, 10 μM) L6 myoblasts were incubated for 10 or 30 min prior to insulin stimulation respectively. H₂O₂ (0.5mM) treatment

was carried out for the indicated time interval. Insulin dose response study was also performed under normal glucose and high glucose with or without 150 nM okadaic acid (serine/threonine phosphatase inhibitor) for the indicated insulin concentrations.

Immunoblotting: L6 myoblasts subjected to various experimental treatments were washed twice with ice-cold phosphate-buffered saline and lysed in RIPA lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 mM sodium butyrate and 0.05% NP-40). After a brief sonication, cells were centrifuged at 12000g and supernatant were collected as total proteins for immunoblotting. Histones were acid extracted using 0.25M HCl according to method described by Tikoo K et al. [27]. Protein concentration was determined by Lowry method [28].

For western blot analysis, proteins were transferred onto nitrocellulose membrane and immunoblot analysis was performed by using Anti p-p38 (rabbit polyclonal 1:1000), Anti p38 (rabbit polyclonal 1:1000), p-MSK1 (rabbit polyclonal, 1:1000), p-p90 RSK2 (rabbit polyclonal, 1:1000), Anti p-Histone H3 (rabbit polyclonal 1:5000), Anti acetylated H3 (rabbit polyclonal 1:5000), Anti monomethyl histone Me-H3-K4 (rabbit polyclonal 1:5000), Anti monomethyl histone Me-H3-K9 (rabbit polyclonal 1:5000) and Anti histone H3 (rabbit polyclonal 1:5000). The antigen–antibody complex was visualized with an ECL detection kit (Amersham Biosciences). The immunoblots were quantified by densitometric scanning with NIH Image J software.

Visualization of Intracellular H₂O₂: Intracellular generation of H₂O₂ was visualized using CM-H2DCF-DA as described by Mahadev K et al. [4]. At 80-90% confluence, L6 myoblasts were treated with either normal glucose (5 mM + 20 mM mannitol) or high glucose (25 mM) for 5 hr and stimulated with insulin (100 nM) with or without prior treatment of DPI (10 μM) and catalase (0.008% w/v). L6 myoblasts were washed with DMEM (without neutral red) and incubated in the dark for 10 min with CM-DCF. Intracellular H₂O₂ production was detected by fluorescence of 5, 6-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA; Cat # C-6827, Molecular Probes) on inverted fluorescence microscope (Nikon, Japan) at an excitation wavelength of 488 nm and emission at 515–540 nm. To avoid photo oxidation, the fluorescence images were collected by a single rapid scan with identical parameters for all samples.

Isolation of total RNA: At 80-90% confluence, L6 myoblasts were treated with 5 mM + 20 mM mannitol in case of normal glucose and 25 mM glucose in case of high glucose for 5 hr and stimulated with insulin (100 nM) for 30 min and at the end of treatment total RNA was isolated. Total RNA was isolated using protocol as describe by Chomczynski et al. [29] using TRIZOL reagent (Invitrogen) and purified using RNeasy kit (Auprep RNeasy mini kit, Life Technologies) according to the manufacturer's protocol. RNA quality and integrity from each sample was assured using Nanodrop (ND-1000) by A260/280 absorbance ratio and using agarose gel electrophoresis respectively. Total RNA prepared from L6 myoblasts was used for cDNA microarray analysis.

Probe labeling and microarray hybridization: The mouse 15K array (Microarray centre, University Health Care, Toronto) used in the present study consisted of 15,264 genes spotted in duplication. Fluorescence labeled cDNA probes were prepared from 20 μ g of total RNA using SuperScript III (Invitrogen Life Technologies), oligo dT primer (Invitrogen Life Technologies) and Cy3 or Cy5 labeled dCTP's (Amersham biosciences) from normal glucose and high glucose insulin treated L6 myoblasts. The Cy3 and Cy5 probes were mixed in equal amount and hybridization was carried out at 43°C for 16 hours on Hyb Array 12 hybridization station (Perkin Elmer). Hybridization was repeated three times. Dye-swapped experiment was performed to improve the accuracy of the measurement and to rule out the non specific signals.

Analysis of microarray data: Slides were scanned with a Scanarray Gx microarray scanner (Perkin Elmer) and the images were analyzed using Scanarray software. Flagged spots were excluded from analysis. The mean value for each spot was taken and analyzed using Avadis software (Strand Lifesciences). The signal intensities were normalized between Cy3 and Cy5 by performing lowess normalization. The \log_2 value for the signal intensity of each spot was calculated for each slide and the significant analysis was performed using student's t test. Fold change was calculated as the ratio of normalized signal intensities of insulin treatment under normal and high glucose conditions. Genes showing more than 2 fold change were taken for further analysis. Genecard (www.genecards.org) and Pubmed (www.pubmed.gov) were used for assigning the genes for specific biological processes.

Statistical analysis: Data are expressed as means \pm S.E.M. Statistical comparison between different groups were done using either student t test for comparison of two groups or for more

than two groups one-way analysis of variance (ANOVA) followed by Tukey test was used. P value less than 0.05 was considered to be significant.

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Results

Insulin potentiates H₂O₂ production under high glucose condition: Increased intensity of DCF green fluorescence was observed by insulin treatment under high glucose condition as compared to normal glucose condition (Figure 1 B & D). However, preincubation with DPI and catalase prior to insulin stimulation subsequently attenuated enhanced production of intracellularly generated ROS by insulin under high glucose condition (Figure 1 E & F). Attenuation of ROS generation with DPI (Nox inhibitor) and catalase (H₂O₂ scavenger) suggests the involvement of NADPH oxidase induced H₂O₂ generation in insulin signaling.

Changes in insulin concentration alters multiple histone modifications: Oxidative stress has been implicated in modulating various histone modifications resulting into chromatin remodeling and change in gene expression [23, 30]. To analyze the role of insulin induced ROS generation under hyperglycemic condition in altering histone modifications, dose response of insulin was performed. Insulin treatment for 30 min showed dose dependent increase in Ser 10 phosphorylation, acetylation and Lysine 4 methylation (Me-H3-K4) of histone H3 under normal glucose condition. In addition, it also showed decrease in Lysine 9 methylation of histone H3. However, there was a dose dependent decrease in histone H3 phosphorylation (Ser10), acetylation, Lysine 4 methylation (Me-H3-K4) and an increase in Lysine 9 methylation (Me-H3-K9) by insulin treatment under high glucose condition (Figure 2). Thus, these results show a complete reversal of insulin induced histone modifications pattern under high glucose condition as compared to low glucose condition.

Insulin under high glucose condition induces dephosphorylation and deacetylation of

Histone H3: To check further the time course of insulin induced alteration in phosphorylation and acetylation of histone H3, L6 myoblasts were stimulated with insulin at 100 nM for different time intervals under normal and high glucose conditions. Treatment of insulin showed time dependant increase in phosphorylation of histone H3 at Ser 10 under normal glucose condition. However, under high glucose condition, insulin treatment resulted in dephosphorylation of histone H3 (Figure 3A). Phosphorylation of histone H3 at Ser10 usually occurs when cell enters in mitosis or during activation of certain genes [31, 32]. Therefore, dephosphorylation of histone H3 by insulin indicates that there might be decrease in insulin induced gene activation under hyperglycemic condition. Moreover, we also observed time dependant dephosphorylation of histone H3 by H₂O₂ similar to insulin under hyperglycemic condition (Figure 3A). These results indicate that insulin mediated intracellularly generated H₂O₂ might be a cause of insulin induced dephosphorylation of histone H3 under hyperglycemic condition. Moreover, similar to dephosphorylation of histone H3, insulin and H₂O₂ treatment also showed deacetylation of histone H3 under high glucose condition (Figure 3C).

Involvement of H₂O₂ in dephosphorylation and deacetylation of histone H3 by insulin under high glucose condition was further confirmed by preincubation of L6 myoblast with catalase and DPI prior to insulin stimulation. Figures 3A & 3C shows attenuation of dephosphorylation as well as deacetylation of histone H3 by DPI and catalase treatment, further emphasizing the role of intracellularly generated H₂O₂ in modulating these histone modifications.

Insulin activates ERK & p38 MAP kinase: Recently, several reports have shown the effect of H_2O_2 on altering histone modifications via activating upstream MAP kinases [23, 24, 27]. Insulin induced activation of MAP kinases, ERK and p38 have been shown to be altered under hyperglycemic condition as a result of generation of reactive oxygen species [7, 9, 33]. Insulin treatment resulted in rapid activation of ERK and p38 as early as 5 min under both normal as well as high glucose condition. However, under normal glucose condition, ERK and p38 activation was sustained for a longer duration (2 hr) while under high glucose condition; it showed rapid deactivation (Figure 4A and 4B). Interestingly, stimulation of L6 myoblasts with H_2O_2 (0.5 mM) also showed rapid activation followed by rapid deactivation of ERK & p38 (Figure 4A and 4B), very similar to insulin under hyperglycemic condition. These results along with earlier observations suggest that level of intracellularly generated H_2O_2 might be involved in dephosphorylation of ERK & p38 by insulin under hyperglycemic condition. To obtain direct evidence for the involvement of H_2O_2 in differential activation of ERK & p38 by insulin under high glucose condition, L6 myoblasts were preincubated with catalase and DPI prior to insulin stimulation. Preincubation of DPI and catalase under hyperglycemic condition resulted in insulin induced sustained activation of p38 similar to insulin treatment under normal glucose condition (Figure 4 A and 4B). These results suggest that enhanced production of H_2O_2 by insulin under hyperglycemic condition is responsible for rapid dephosphorylation of ERK and p38.

Activation of RSK2 and MSK1 by insulin and H_2O_2 : RSK2 and MSK1 are the known downstream histone kinases that are found to be activated by ERK and p38 MAP kinases in response to growth factors, mitogens and various stress stimuli [15, 16]. To explore, whether, activation of ERK/p38 MAPK by insulin and H_2O_2 coincide with activation of downstream

histone kinases, phosphorylation status of RSK2 and MSK1 was investigated. Insulin induced activation of RSK2 and MSK1 under both normal as well as high glucose condition was similar to upstream kinases (ERK and p38) (Figure 4C and 4D). Furthermore, there was rapid deactivation of RSK2 and MSK1 by insulin under hyperglycemic condition. Treatment with catalase and DPI prevented insulin mediated rapid deactivation of RSK2 and MSK1 under hyperglycemic condition. H₂O₂ treatment also showed activation pattern of RSK2 and MSK1 similar to insulin treatment under hyperglycemic condition (Figure 4C and 4D).

In spite of activated levels of RSK2 and MSK1 up to 40 minutes of insulin treatment under high glucose conditions, our data showed dephosphorylation of histone H3 within 10 minutes of insulin stimulation (Figure 3 and 4). These results suggest that insulin induced dephosphorylation of histone H3 under hyperglycemic condition could be either independent of these upstream kinases or may involve histone phosphatases.

Okadaic acid prevents insulin induced change in phosphorylation and acetylation of histone H3: It has been reported earlier that hyperglycemia associated with insulin resistance can lead to activation of cellular phosphatases which are involved in deactivation of various kinases [8, 34, 35]. In addition, there are also certain reports that show the role of serine/threonine phosphatases in regulating histone phosphorylation [17, 18, 36]. To check the involvement of these serine/threonine phosphatases in modulating insulin induced multiple histone modifications, L6 myoblasts were treated with okadaic acid (serine/threonine phosphatase inhibitor). Preincubation with okadaic acid inhibited insulin induced dephosphorylation of histone H3 under high glucose condition (Figure 5). Indicating that insulin induced changes in the histone phosphorylation at Ser10 may be serine/threonine phosphatase

dependant. Okadaic acid also prevented insulin induced deacetylation of histone H3, suggesting a crosstalk between histone phosphorylation and acetylation. However, we failed to observe any change in methylation of histone H3 at Lysine 4 and Lysine 9 by insulin in the presence of okadaic acid (Figure 5); suggesting histone methylation may be independently regulated.

Changes in global gene expression by insulin under high glucose condition: Insulin induced alterations in multiple histone modifications under high glucose condition suggests alteration in global gene expression. This was confirmed by carrying out cDNA microarray analysis in L6 myoblast by insulin treatment under normal and high glucose conditions. We observed that insulin induces downregulation of 58% genes out of 469 genes that showed ≥ 2 fold changes in gene expression under high glucose condition (see supplementary data). In microarray data analysis, genes which are significantly down regulated correspond to signal transduction, transcription, metabolism, protein transport, cell adhesion and ion transport (Figure 6). In addition to this, our results also showed up regulation of stress responsive and chromatin modification genes which further support our data.

Discussion

Hyperglycemia with increased production of reactive oxygen species has been shown to alter insulin signaling in skeletal muscle and adipocytes in diabetes [37, 38]. Insulin has been shown to generate intracellular H_2O_2 under hyperglycemic condition which modulates post receptor insulin signaling cascade [4-6, 39].

In the present study, we report for the first time that insulin stimulation enhances the production of ROS which alters histone H3 modifications under hyperglycemic condition. Our data shows enhanced production of intracellular ROS by insulin under high glucose condition. Pre-incubation of L6 myoblasts with DPI (NADPH oxidase inhibitor) and catalase (H_2O_2 scavenger) attenuates ROS generation, suggesting involvement of NADPH oxidase in insulin induced H_2O_2 generation. Recently, Hansen et al., have reported inhibition of insulin signaling by micromolar concentrations of H_2O_2 whereas Mahadev et al., have shown increase in distal insulin signaling as result of insulin induced ROS generation [4, 8]. These results suggest that ROS can exert different effects on insulin signaling depending upon its concentration in a particular cellular condition. The amount of ROS is dependent on the type of cell and its mitochondrial activity. Mitochondrial mass is reported to be increased in myotubes as compared to myoblasts [40]. Thus, the effect of enhanced production of intracellular ROS by insulin under high glucose condition may vary from one cell type to other and needs to be checked further.

Insulin induces increase in phosphorylation and acetylation of histone H3 under normal glucose condition in a time dependant manner. However, this histone modifications pattern was completely reversed under high glucose condition. Moreover, incubation with H_2O_2 also shows similar pattern of phosphorylation and acetylation of histone H3 by insulin treatment under high glucose condition. Thus, emphasizing the involvement of insulin induced generation of ROS in

modulating post translational modifications of histone H3. These observations suggest that changes in glucose concentration, not only affects the level of insulin induced ROS generation but also affects the post-translational modifications of histone H3.

Mitogen activated protein kinases, ERK and p38 MAPK has been shown to be associated with phosphorylation of histone directly or via activation of downstream kinases such as RSK2 and MSK1 [15, 16]. Growth factors, mitogens and various stress stimuli have been reported to induce phosphorylation of histone H3 via activation of these kinases [15, 41, 42]. Our data also show rapid activation of ERK, p38 MAPK, RSK2 and MSK1 by insulin under both normal as well as high glucose conditions. Under normal glucose condition, insulin treatment shows sustained phosphorylation of these kinases whereas under high glucose condition, there is a rapid decrease in phosphorylation of these kinases. However, at early time points despite of activation of these histone kinases, we observed rapid dephosphorylation of histone H3 under hyperglycemic condition thus indicating a paradoxical situation. Suggesting dephosphorylation of histone H3 by insulin treatment under hyperglycemic condition may either be independent of histone kinase or may involve activation of histone phosphatases.

Several reports have shown the involvement of serine/threonine phosphatases in regulating serine 10 histone H3 phosphorylation [17, 18, 36]. Role of histone phosphatases on insulin induced histone H3 modifications was further confirmed by pretreatment with okadaic acid. Pretreatment of okadaic acid inhibited insulin induced dephosphorylation and deacetylation of histone H3 under high glucose condition. Evidently, these observations provide indirect evidence that serine/threonine phosphatases might be playing a key role in dephosphorylation and deacetylation of histone H3. However, our microarray data supports involvement of serine/threonine phosphatases (PP1 and PP2A) in insulin induced dephosphorylation of histone

H3. PP1 and PP2A expression were up regulated by 1.5 and 1.1 fold respectively by insulin treatment under hyperglycemic condition (unpublished observation).

Phosphorylation and acetylation of histone H3 has been shown to be associated with gene activation. In addition to this, Lysine 4 methylation of histone H3 is also shown to be involved in gene activation whereas, Lysine 9 methylation of histone H3 is associated with gene repression [25, 26]. Our data shows that increase in insulin concentration decreases methylation of histone H3 with concurrent increase in Lysine 9 methylation. Thus, these results demonstrates the global down regulation of insulin induced gene expression under hyperglycemia and this was further confirmed by microarray data showing global down regulation of genes belonging to variety of biological processes.

In summary, our results demonstrate that insulin induced intracellular generated oxidative stress is involved in modulating multiple histone modifications under hyperglycemic conditions. Our results also reveal that phosphorylation of histone H3 at Ser 10 is independent of known histone kinases and suggest the role of serine/threonine phosphatase in modulating insulin signaling. Further, studies are required to conclude role of phosphatase and its inhibitor in diabetes.

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Legends for Figure

Figure 1 Insulin-stimulated production of H₂O₂ in L6 myoblasts under normal and high glucose condition with or without preincubation of DPI and catalase: L6 myoblasts were cultured as described in “Materials and Methods” and stimulated with 100 nM insulin for 10 min. In case of DPI and catalase, L6 myoblasts were preincubated with 10 μM DPI for 30 min or with catalase (0.008%) for 10 min prior to insulin stimulation. Representative figures of phase contrast and DCF fluorescence of different treatment groups where; **A and a:** normal glucose, **B and b:** normal glucose + insulin, **C and c:** high glucose, **D and d:** high glucose + insulin, **E and e:** high glucose + catalase + insulin and **F and f:** high glucose + DPI + insulin. Results shown are representative of three different experiments. **A, B, C, D, E, F** are DCF fluorescence images and **a, b, c, d, e, f** are respective phase contrast images.

Figure 2 Concentration dependant changes in histone modifications by insulin: L6 myoblasts were cultured as described in “Materials and Methods” and treated with different concentrations of insulin as indicates for 30 min under normal glucose and high glucose conditions. At the end of treatment, L6 myoblasts were washed with ice cold PBS, lysed with lysis buffer and processed for immunoblotting using specific antibody. **A, B, C and D:** depict immunoblots and respective densitometry quantified bar graphs of histone p-H3 (Ser 10), Ac-H3, Me-H3-K4 and Me-H3-K9 respectively of insulin treatment under normal and high glucose conditions. **E:** Immunoblot of histone H3 was used as a control to normalize p-H3 (Ser 10), Ac-H3, Me-H3-K4 and Me-H3-K9. Values are expressed as mean ± S.E.M of three independent experiments. In the graphs, symbols indicate statistical comparison * Vs control NG-Ins, § Vs control HG-Ins and # Vs respective NG-Ins. p values obtained after pair wise comparison ***

$p < 0.001$, ** $p < 0.01$, * $p < 0.05$, § $p < 0.05$, ### $p < 0.001$, ## $p < 0.01$ and # $p < 0.05$. **NG**: normal glucose, **HG**: high glucose, **Ins**: insulin.

Figure 3 Time course of Insulin and H₂O₂ on phosphorylation and acetylation of histone H3 in L6 myoblasts: L6 myoblasts were cultured as described in “Materials and Methods” and stimulated with 100 nM insulin or H₂O₂ (0.5 mM) for indicated time. At the end of treatment, L6 myoblasts were washed with ice cold PBS, lysed with lysis buffer and processed for immunoblotting using specific antibodies. In case of DPI and catalase under high glucose condition, L6 myoblasts were preincubated for 30 min with 10 µM DPI or with catalase (0.008%) for 10 min prior to insulin stimulation. Immunoblots of histone p-H3 (Ser10) and Ac-H3, and H3 under different condition have been shown above where; **A**: immunoblots of p-H3, **B**: densitometry quantified line graph of p-H3. **C**: immunoblots of Ac-H3, **D**: densitometry quantified line graph of Ac-H3 and **E**: immunoblots of histone H3. **120'**, 120 min control without insulin. Values are expressed as mean ± S.E.M of three independent experiments. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ and §§§ $p < 0.001$, *vs. normal glucose, § vs. high glucose. **NG**: normal glucose, **HG**: high glucose, **Ins**: insulin, **DPI**: diphenylene iodonium and **Cat**: catalase

Figure 4 Time course of insulin and H₂O₂ on phosphorylation of ERK, p38, MSK1 and RSK2 in L6 myoblasts: L6 myoblasts were cultured as described in “Materials and Methods” and stimulated with 100nM insulin or H₂O₂ (0.5 mM) for the indicated time. At the end of treatment, L6 myoblasts were washed with ice cold PBS and lysed with lysis buffer and processed for immunoblotting using specific antibodies. In case of DPI and catalase, L6 myoblasts were preincubated for 30 min with 10 µM DPI or with catalase (0.008%) for 10 min

prior to insulin stimulation. Representative immunoblots of p-ERK, p-p38, p-MSK1, p-RSK2 and p38 used as a loading control under different treatment conditions, where; **A**: Immunoblots of p-ERK, **B**: Immunoblots of p-p38, **C**: Immunoblots of p-RSK2 and **D**: Immunoblots of p-MSK1. **E**: Immunoblots of p38. Densitometry analyzed bar graphs of these immunoblots are shown in supplementary data. Results shown are representative of three different experiments. **NG**: normal glucose, **HG**: high glucose, **Ins**: insulin, **DPI**: diphenylene iodonium and **Cat**: catalase

Figure 5 Effect of serine/threonine phosphatases on insulin induced changes in histone modifications under hyperglycemic condition: L6 myoblasts were cultured as described in “Materials and Methods” and treated with okadaic acid (150 nM) prior to insulin stimulation at different concentrations for 30 min under high glucose condition. At the end of treatment, L6 myoblasts were washed with ice cold PBS, lysed with lysis buffer and processed for immunoblotting using specific antibodies. **A**: representative immunoblots of p-H3, Ac-H3, Me-H3-K4 and Me-H3-K9 and histone H3. **B, C, D, and E**: depicts densitometry quantified bar graph of p-H3, Ac-H3, Me-H3-K4 and Me-H3-K9 after normalized with histone H3 (for comparison, bar graph was drawn using immunoblot data from figure 2 and 5). Values are expressed as mean \pm S.E.M of three independent experiments. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ these are the p values obtained after pairwise comparison with corresponding HG-Ins.

Figure 6 Microarray analysis of insulin under high glucose condition on global changes: Insulin induced up and down regulation of numerous genes associated with various biological processes under high glucose condition in L6 skeletal muscle myoblasts. (Figure derived from

supplementary data and assign to corresponding biological process as describe in “Materials and Methods”)

ACCEPTED MANUSCRIPT

Figure 1

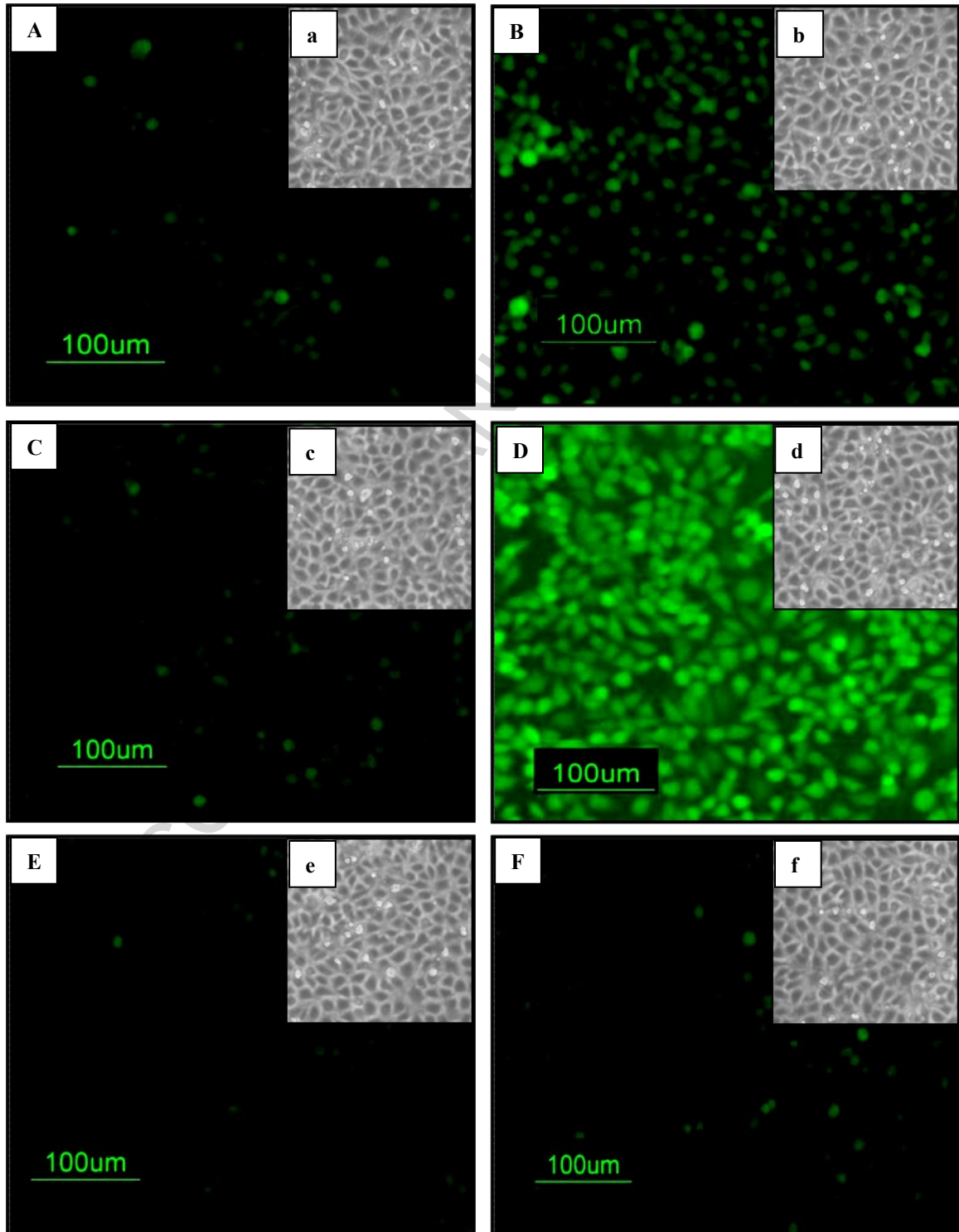
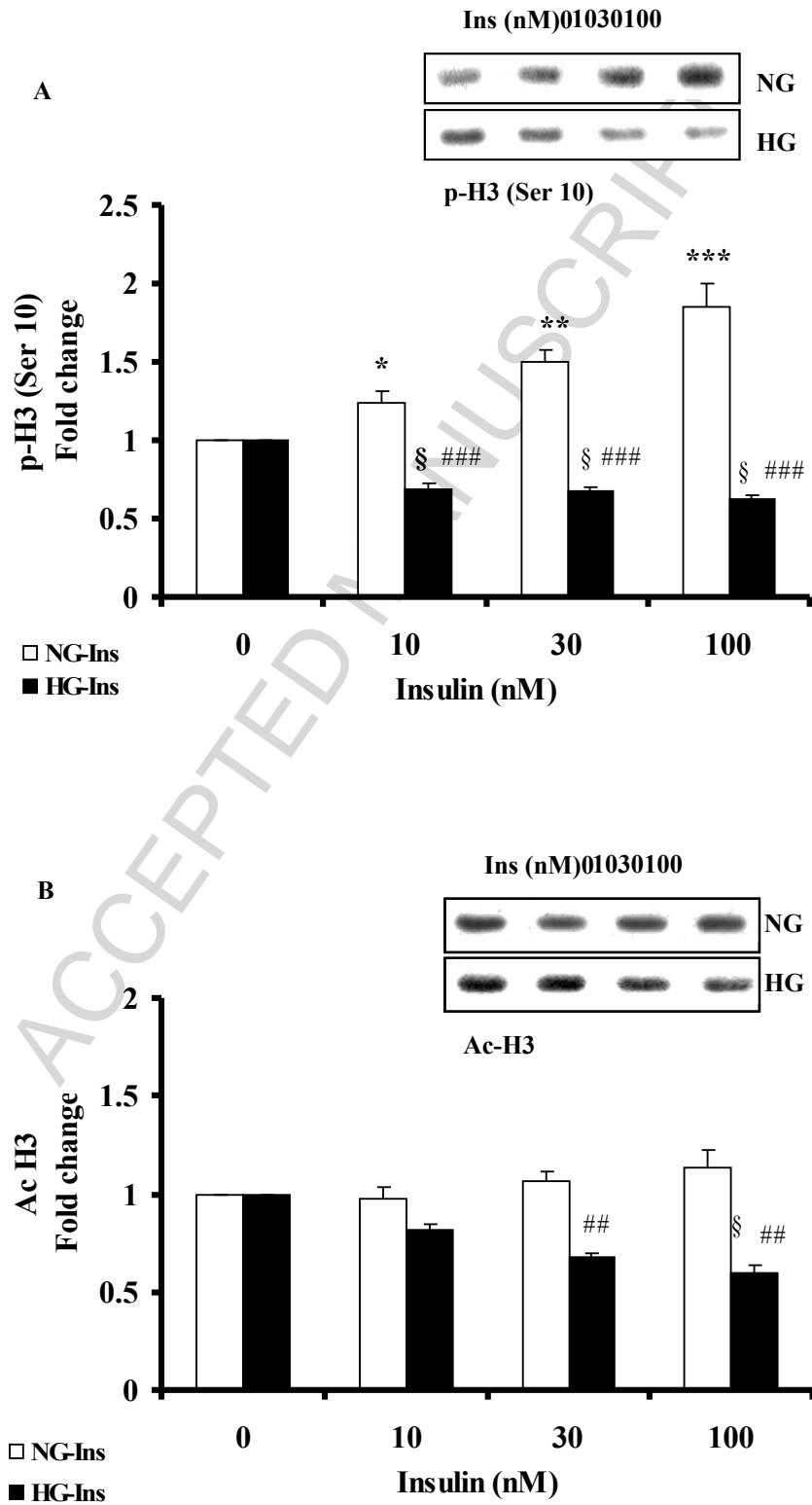


Figure 2



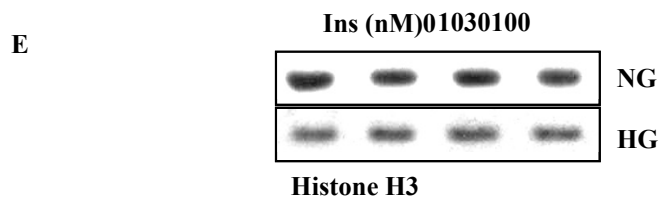
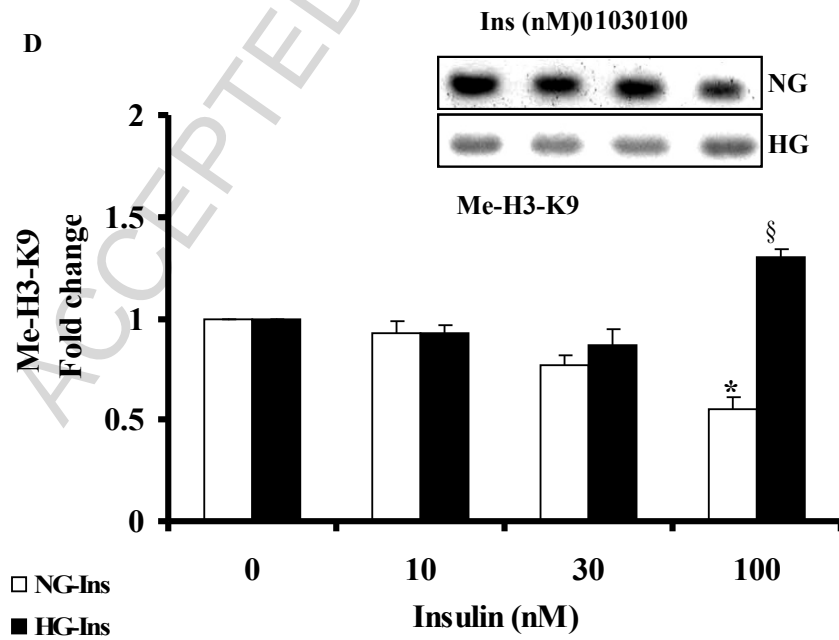
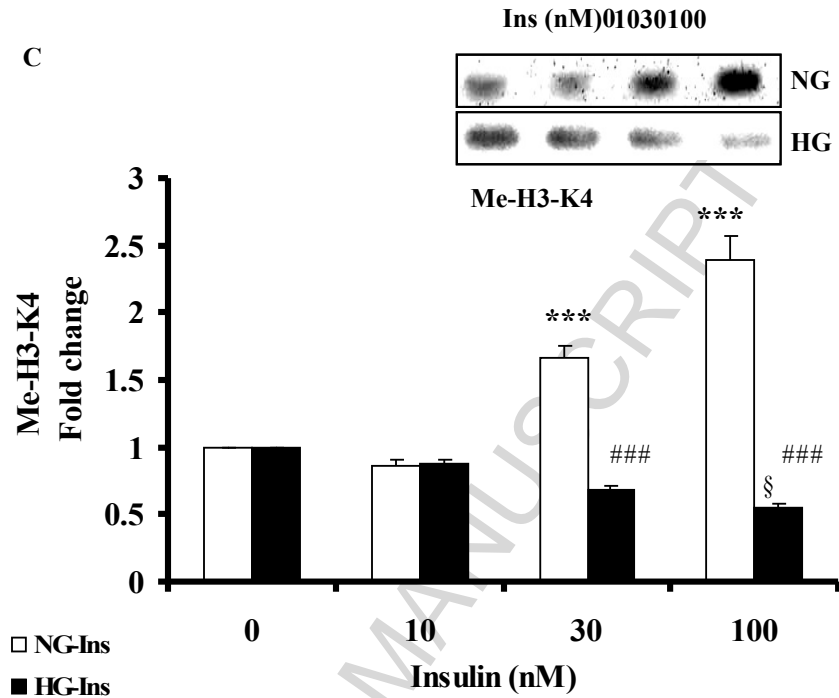
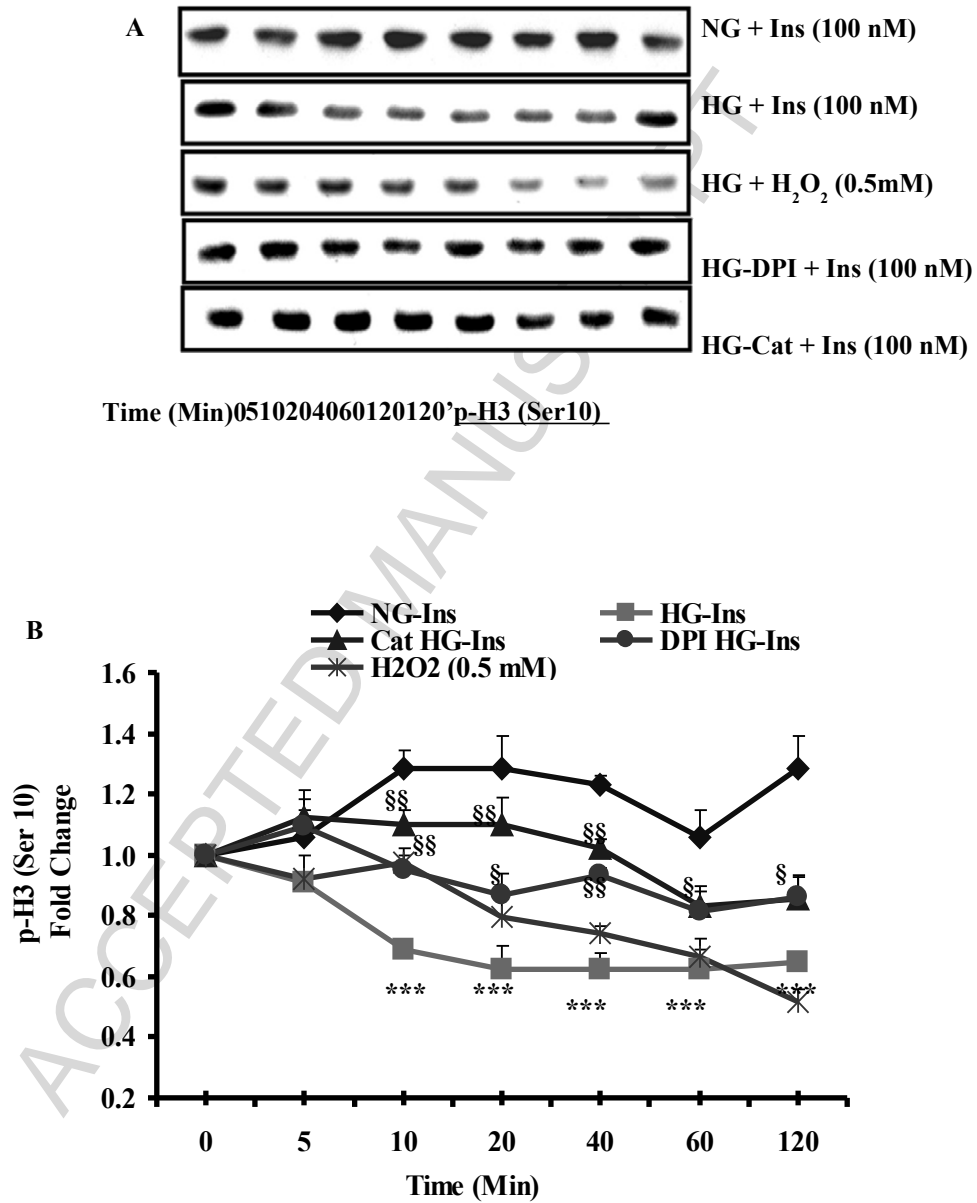
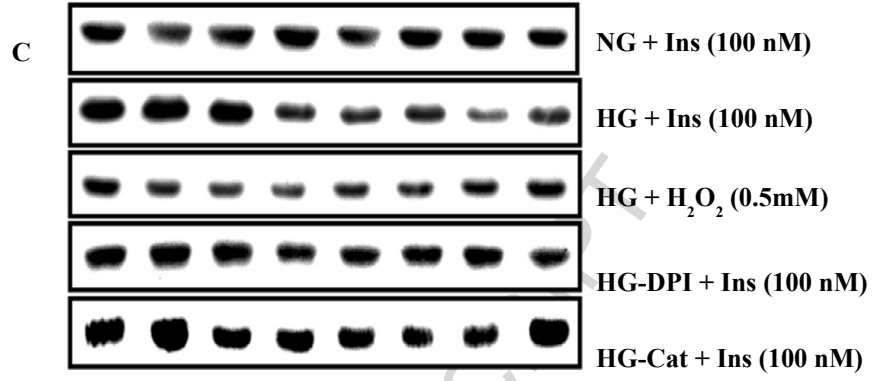
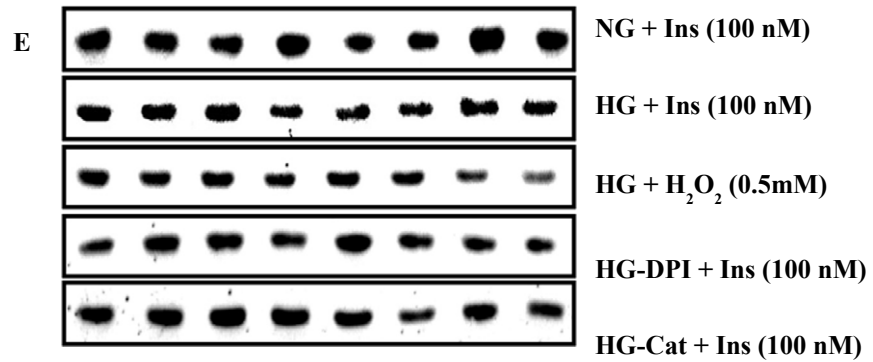
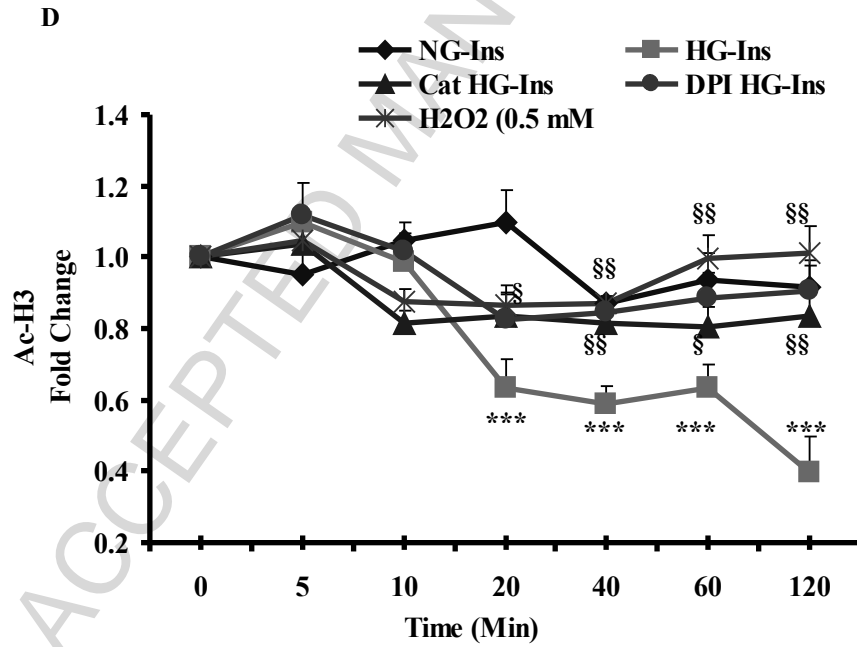


Figure 3





Time (Min) 0 5 10 20 40 60 120 Ac-H3



Time (Min) 0 5 10 20 40 60 120 Histone H3

Figure 4

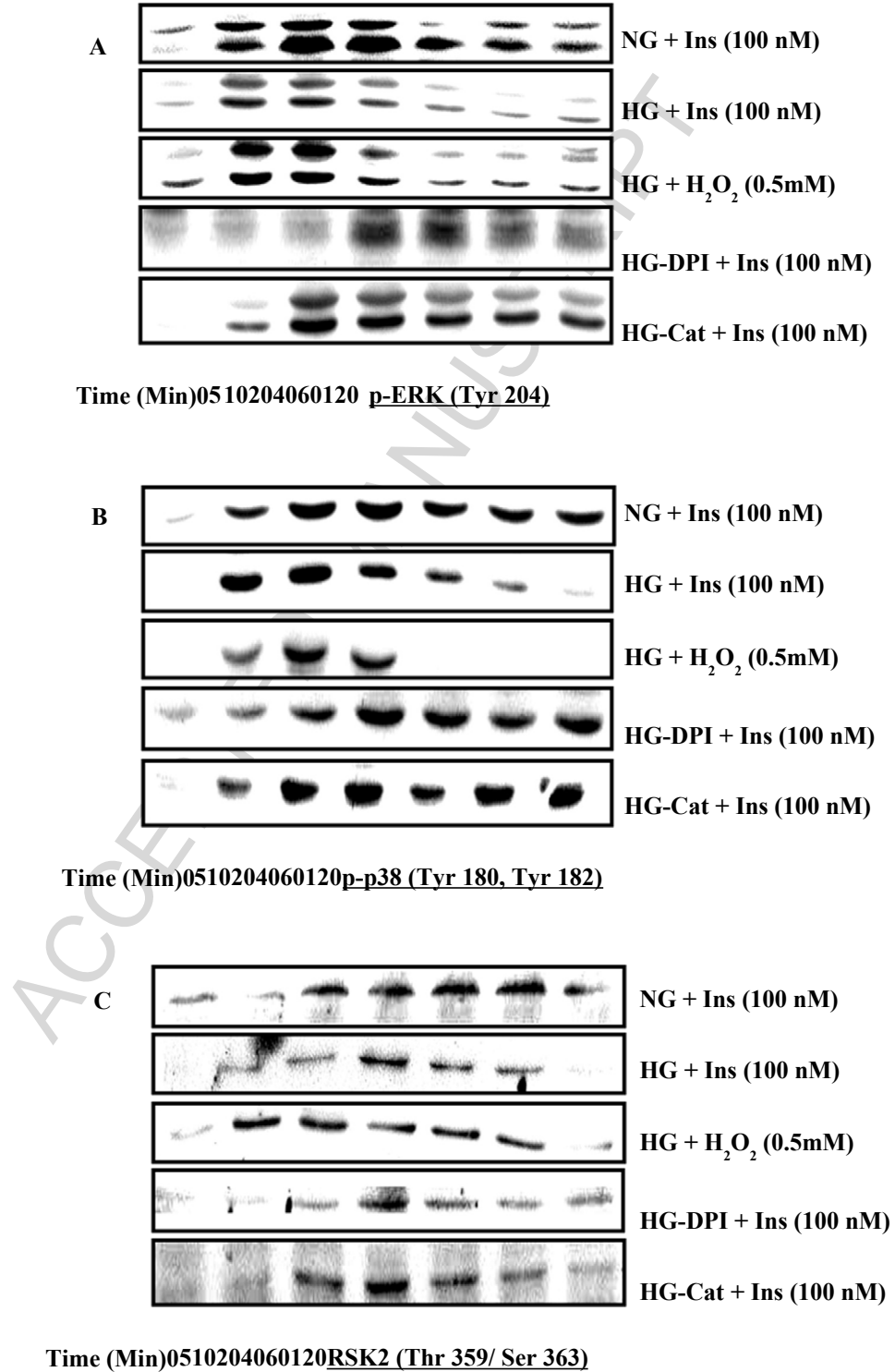


Figure 4 continue

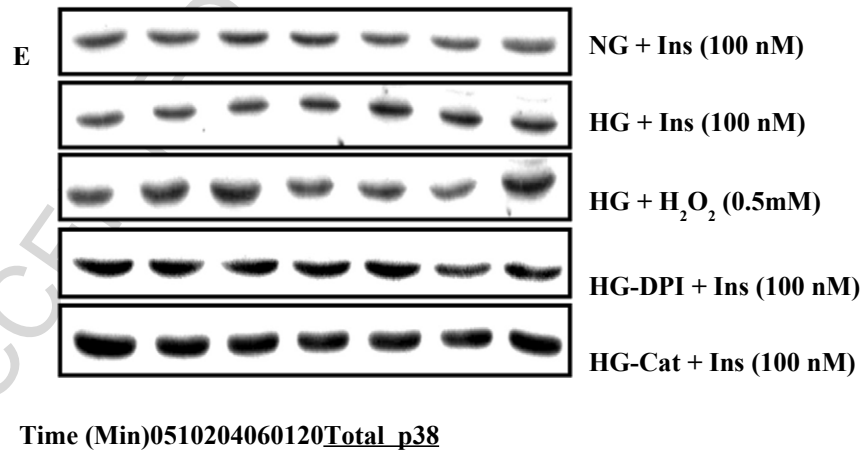
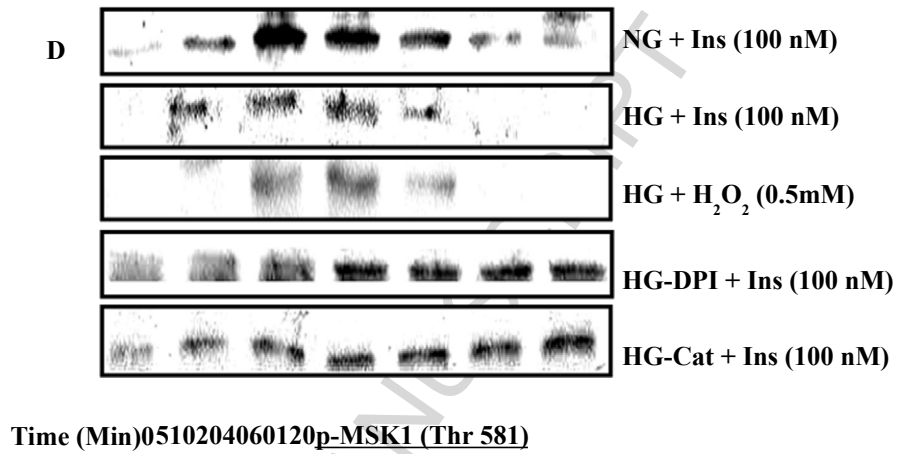
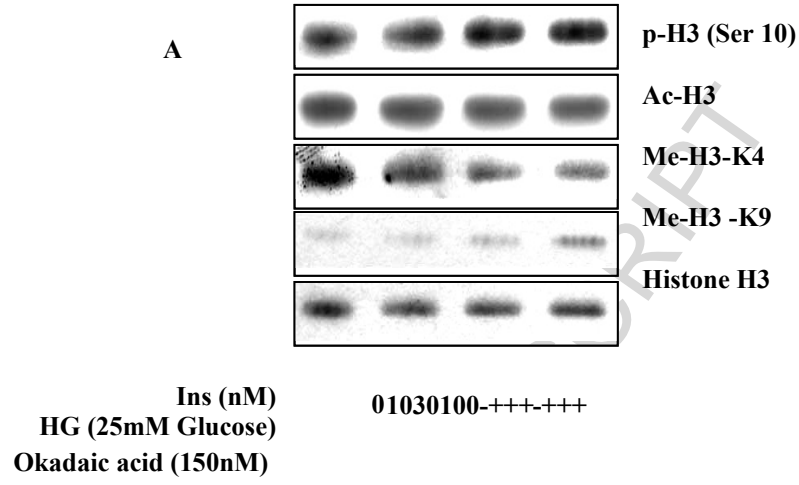


Figure 5



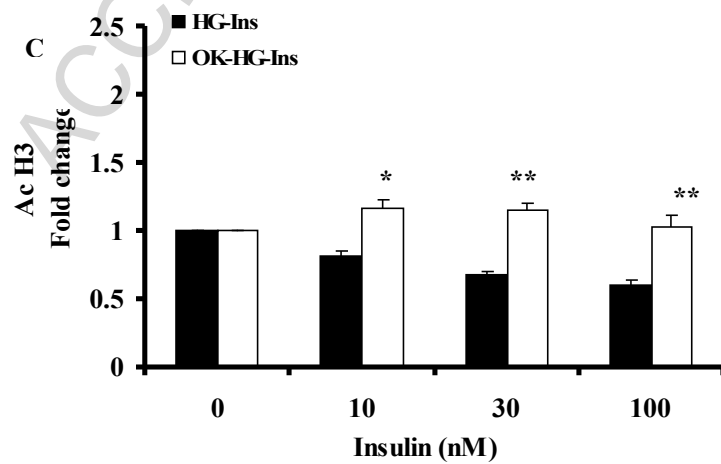
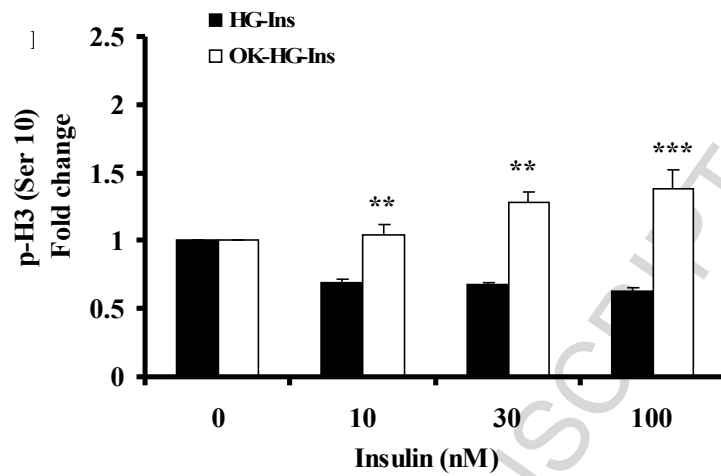


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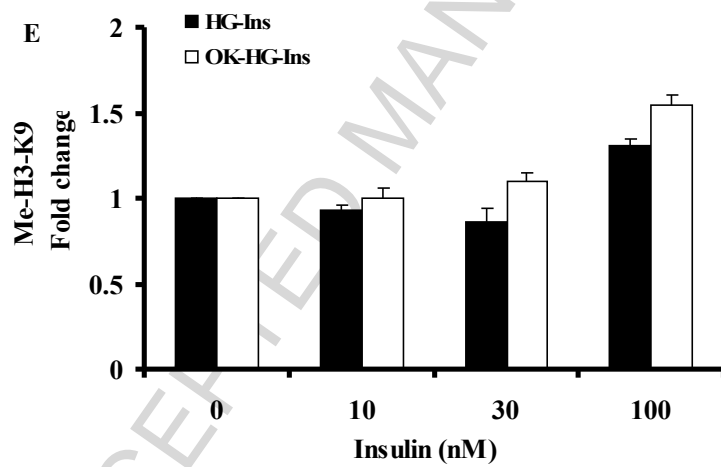
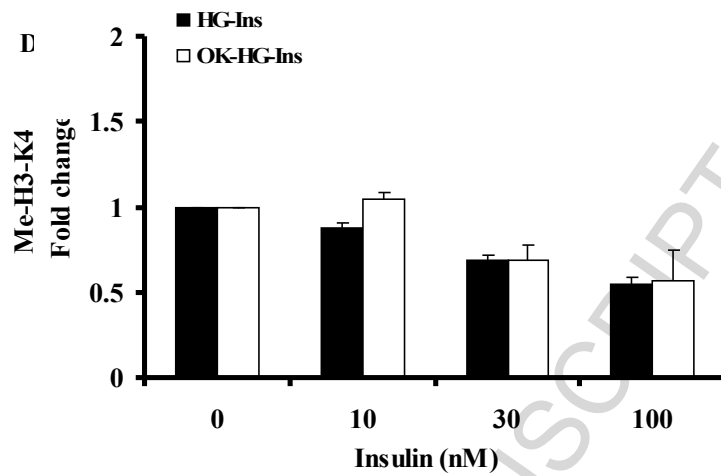


Figure 6

