

# Trivalent Chromium Modulates Hexosamine Biosynthesis Pathway Transcriptional Activation of Cholesterol Synthesis and Insulin Resistance

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## ABSTRACT

Trivalent chromium has long been recognized to benefit carbohydrate and lipid metabolism. Given emerging evidence that suggests chromium improves insulin sensitivity through the maintenance of an optimal level of plasma membrane (PM) cholesterol, we delineated the role of this micronutrient in attenuating hyperinsulinemia-induced cholesterol biosynthesis and insulin resistance. Exposing 3T3-L1 adipocytes to physiological hyperinsulinemia (500 pM 12 h), resulted in a marked impairment in insulin-stimulated glucose transport. Concurrent treatment with chromium in the picolinate form (CrPic, 10 nM 16 h) prevented against glucose transport dysfunction. Insulin signaling was neither impaired by hyperinsulinemia nor amplified by chromium to promote this protective action. Instead, it was found that hyperinsulinemia promoted an increase in PM cholesterol content that was observed to impair the acute ability of insulin to stimulate GLUT4 redistribution to the PM. Chromium prevented against the accumulation of PM cholesterol. Mechanistically, hyperinsulinemia promoted increases in *O*-GlcNAc modification of specificity protein 1 (Sp1), known to engage a cholesterolgenic response. Subsequent chromatin immunoprecipitation and luciferase assays revealed that hyperinsulinemia increased the binding affinity of Sp1 to the promoter region of *Hmgcr*, encoding 3-hydroxy 3-methyl-glutaryl-CoA reductase (HMGR), as well as HMGR promoter activity. This resulted in gains in mRNA and protein content of HMGR, with resulting elevations in PM cholesterol content. Moreover, treatment with chromium prevented this transcriptional response. Together, these data suggest a mechanism whereby CrPic affords glycemic health through inhibition of a transcriptional cholesterolgenic program detrimental to insulin action.

**Keywords:** 3T3-L1 Adipocytes; GLUT4; HMG-CoA Reductase; Hyperinsulinemia; Sp1

## 1. Introduction

Clinical trials have revealed beneficial actions of trivalent chromium ( $\text{Cr}^{3+}$ ) on glycemic control [1-3], yet a mechanism of action remains unknown. In this regard, data collected in our lab as well as others revealed that  $\text{Cr}^{3+}$  exerts its influence on PM parameters [4-6]. Interestingly, these studies suggested that regulation of glucose transport by  $\text{Cr}^{3+}$  may be independent of amplification of insulin signaling, as previously described [7]. Rather, it was found that  $\text{Cr}^{3+}$  normalized elevated PM cholesterol levels that impaired glucose transport in 3T3-L1 adipocytes cultured under the diabetic milieu. Exogenous add back of cholesterol, for instance, blunted the effects of  $\text{Cr}^{3+}$  observed in these insulin-resistant cells. Despite this association in clonal cells, a basis for alterations in PM cholesterol content in promoting the development of

insulin resistance remained unclear.

Parallel experiments have since established that nutrient overabundance promotes elevations in membrane cholesterol content in 3T3-L1 adipocytes and L6 myotubes, as well as in skeletal muscle of C57Bl/6J mice and Zucker rats [8-12]. These membrane perturbations were observed concomitant with a loss of cortical filamentous-actin (F-actin) necessary for proper incorporation of the insulin sensitive glucose transporter GLUT4 into the PM. Furthermore, increased membrane cholesterol content was also correlated with diminished glucose disposal rates in humans [10]. Mechanistically, data revealed increased glucose flux through the hexosamine biosynthesis pathway (HBP) promoted elevated *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification of specificity protein 1 (Sp1), leading to transcriptional activation of HMG-CoA

reductase, the rate limiting enzyme in cholesterol synthesis [12]. This culminated in increased PM cholesterol content that perturbed F-actin structure and impinged upon insulin action. Inhibition of the HBP or Sp1 binding to the DNA attenuated hyperinsulinemia-induced PM cholesterol accumulation, F-actin loss, and insulin resistance. Strikingly, these increases in cholesterol synthesis were independently found to impair cholesterol efflux processes that HBP inhibition also corrected [13].

Based on these studies finding PM stress compromised insulin action, we hypothesized that  $\text{Cr}^{3+}$  may potentially counter excessive HBP activity thereby inhibiting a transcriptional response leading to dysregulated cholesterol synthesis. We report herein that  $\text{Cr}^{3+}$  protects against hyperinsulinemia-induced increases in PM cholesterol content through inhibition of this pathway. These studies highlight a novel protective role of this micronutrient on glucose metabolism entailing the maintenance of optimal membrane cholesterol content.

## 2. Materials and Methods

### 2.1. Cell Culture and Treatments

Murine 3T3L1 adipocytes purchased from Dr. Howard Green were cultured as described previously [14]. Briefly, preadipocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose and 10% bovine calf serum at 37°C at 10%  $\text{CO}_2$ . Confluent fibroblasts were induced to differentiate as previously described [8]. All studies were performed on adipocytes between 8 and 12 days post initiation of differentiation. For treatments, cells were left untreated or treated with CrPic (10 nM, 16 h Nutrition 21) in the presence or absence of hyperinsulinemia (500 pM, 12 h Sigma) in serum free DMEM. Acute insulin stimulations were performed with a 100 nM dose during the last 5 min for signaling or 30 min for glucose transport analyses.

### 2.2. Glucose Uptake

Glucose uptake assays were performed as described previously [12]. Briefly, treated cells were incubated in a KRPH buffer (136 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM sodium phosphate, pH 7.4, 4.7 mM KCl, 1 mM  $\text{MgSO}_4$ , 1 mM  $\text{CaCl}_2$ ) for 15 min. Cells were then left unstimulated or stimulated with 100 nM insulin for 30 min and exposed to 50  $\mu\text{M}$  2-deoxyglucose (2-DG) containing 0.5  $\mu\text{Ci}$  2- $^3\text{H}$  deoxyglucose (Perkin Elmer) in the absence or presence of 20  $\mu\text{M}$  cytochalasin B. After 10 min, uptake was terminated by aspiration and quenched with the addition of 1.0 ml of 10  $\mu\text{M}$  cytochalasin B. Cells were solubilized in 0.2 N NaOH and  $^3\text{H}$  was measured by liquid scintillation. Counts were normalized to

total cellular protein, determined by the Bradford method.

### 2.3. Protein Analyses

Whole cell lysates were prepared following treatments as previously described [12]. 30 - 50  $\mu\text{g}$  of lysates were resolved and immunoblotted with antibodies to HMGR (Millipore), p-Akt (Genscript) Akt2 (Cell Signaling), p-Akt substrate of 160 kilodaltons (Cell Signaling), and AS160 (Upstate). Immunoblots were quantitated using a LI-COR Odyssey infrared imaging system, normalizing signal intensity to  $\beta$ -actin (Cytoskeleton). For immunoprecipitation, 1.5 mg of protein and 2  $\mu\text{g}$  of Sp1 antibody (Santa Cruz) were used as previously described [12]. Eluted immunoprecipitates were then resolved and immunoblotted using an RL2 antibody (Thermo Scientific).

### 2.4. Cholesterol Measurements

For cholesterol measurements, purified PM fractions were obtained as described [12] and cholesterol content was assayed using the Amplex Red cholesterol assay kit (Molecular Probes). Briefly, reconstituted PM pellets were vigorously mixed with chloroform-methanol (2:1 v/v) for 10 min to extract the cholesterol. The mixture was then centrifuged at 1000 rpm for 10 min. The lower phase containing lipids was then evaporated at 100°C for 10 min. The residue was reconstituted with isopropanol-Triton X solution (10:1 v/v) and 50  $\mu\text{l}$  of sample was combined with 50  $\mu\text{l}$  of Amplex Red reaction mix and incubated at 37°C for 30 min. After incubation, absorbance was measured at 600 nm.

### 2.5. Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed on treated adipocytes as described previously [12]. Briefly, adipocytes were fixed with 1% formaldehyde for 10 min. Cells were then scraped in PBS plus protease inhibitor cocktail and centrifuged for 2 min at 2000 rpm. The pellet was resuspended in 500  $\mu\text{l}$  ChIP lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS plus protease inhibitor cocktail), sonicated (10 pulses of 30 s on, 30 s off), and centrifuged at 14,000 rpm at 4°C for 10 min. Fragmented chromatin preparations were then diluted with ChIP dilution buffer (Millipore), and an input sample was collected. Samples were precleared prior to immunoprecipitation with antibodies to Sp1 (Santa Cruz) or IgG (Millipore) overnight. Eluates were reversed cross-linked and purified DNA was recovered using the phenol/chloroform method. DNA was subsequently used as a template for qPCR under the following conditions: 15 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 50°C, and 34 s at 60°C. The % input method was used to quantitate cycle threshold (Ct) values. The sequence of

the forward primer was (5'-3') ACCCGTCA-TTGGTTGGCTCT and reverse primer (5'-3') CTCCT-AACAACCGCCAAC.

## 2.6. Plasmids and Luciferase Assays

The proximal promoter sequence of HMGR (-284 to +36), required for high level expression, was amplified from mouse genomic DNA using PCR [15,16]. Promoter fragments were sequenced and cloned into pGL2B luciferase reporter plasmids (Promega, Madison WI). Differentiated adipocytes were electroporated (0.16 kV and 960  $\mu$ F) as previously described [12]. Briefly, cells were trypsinized and pelleted by centrifugation at 1000 rpm. Pellets were resuspended in PBS and centrifuged. Pellets were then resuspended in 0.5 ml PBS. For transfection, 50  $\mu$ g of HMGR pGL2B and 50  $\mu$ g of phrlmin TK (Renilla) plasmid were used with a concentration of approximately  $1 \times 10^7$  cells/0.5 ml. A single pulse was applied using a Gene Pulser (Bio-Rad #1652076). The electroporated cells were then allowed to recover and plated into a 24 well plate. Treatments were begun 24 h after electroporation. After treatments, cells were lysed and assayed for promoter activity using the dual luciferase reporter assay system (Promega, Madison WI). Firefly Luciferase activities were normalized to Renilla activity to control for differences in transfection efficiency.

## 2.7. RNA Analyses

Adipocyte RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's protocols. RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Subsequent qPCR reactions were performed under the following conditions: 15 min at 95°C followed by 40 cycles of 15 s at 95°C and 40 s at 60°C. Ct values were normalized to 36B4. The  $\Delta\Delta$ Ct method was used to determine relative expression levels. The sequence for the HMGR forward primer was (5'-3') TGTGGGAA-CGGTGACACTTA and reverse primer (5'-3') CTC-AAATTTTGGGCACTCA. The sequence of for the 36B4 forward primer was (5'-3') AAGCGCGTCCT-GGCATTGTCT and reverse primer (5'-3') CCGCAGG-GGCAGCAGTGGT.

## 2.8. Statistical Analyses

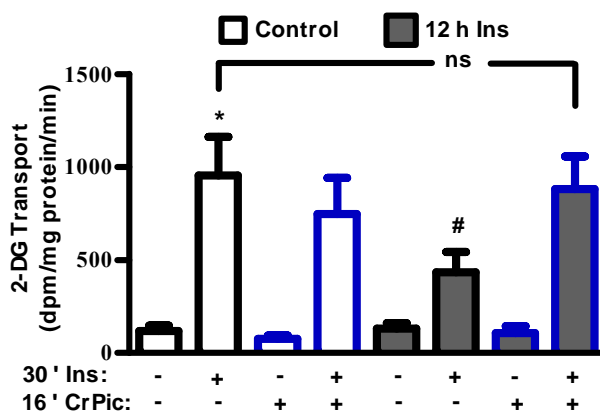
Values presented are means  $\pm$ SEM. The significance of difference between means was evaluated by repeated measures ANOVA. Where a difference was indicated by ANOVA, a Newman-Keuls *post-hoc* test was used to compare differences between groups. Statistical comparisons of the fold or percent change of HMGR expression and Sp1 O-linked glycosylation were performed by two-

tailed Student's *t* test analysis. GraphPad Prism 5 software was used for all analyses.  $P < 0.05$  was considered significant.

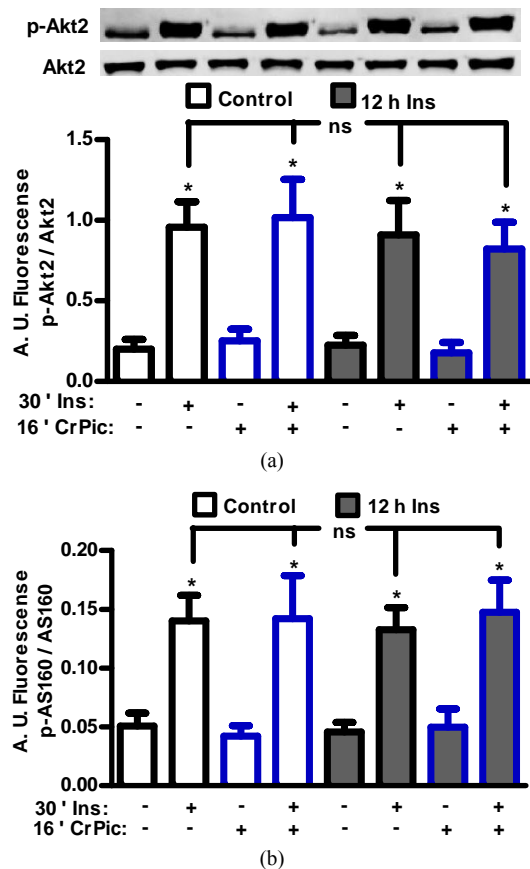
## 3. Results

### 3.1. CrPic Prevents Impaired Glucose Transport

Study first sought to examine the beneficial effects of CrPic treatment on cells rendered insulin resistant. Low doses of pathophysiologic hyperinsulinemia, utilized by many groups to induce insulin resistance [17-20], resulted in a 50% reduction in the ability of a maximal dose of insulin to stimulate glucose transport into adipocytes (**Figure 1**). In cells treated with hyperinsulinemia in the presence of a low, pharmacologically relevant dose (10 nM) of CrPic for 16 h [4,6], the acute ability of insulin to stimulate glucose transport was corrected. Studies have suggested that a beneficial aspect of CrPic on glucose transport may entail countering defects in insulin signaling, thereby improving GLUT4 translocation to the PM [21-24]. Importantly, cell systems that mimic pathophysiological hyperinsulinemia, known to promote the progression/worsening of insulin resistance, and CrPic, known to improve insulin action, are not associated with insulin signaling changes [4,6,11,17]. We thus next sought to verify that insulin signaling was indeed intact under hyperinsulinemic conditions and that the beneficial effect of CrPic was not coupled to enhancement in insulin signaling in these cells. Neither hyperinsulinemia, nor CrPic had any effects on signaling to Akt or its downstream target, AS160. Combinatorial treatment also did not affect any signaling parameters examined (**Figure 2**).



**Figure 1.** CrPic improves insulin responsiveness rendered impaired by hyperinsulinemia. 3T3-L1 adipocytes were treated with or without 10 nM CrPic for 16 h and 500 pM insulin for 12 h. After treatments, 3T3-L1 adipocytes were left unstimulated or stimulated by a maximal dose (100 nM) of insulin for 30 min to initiate glucose uptake. Values are mean  $\pm$  SEM from 4 - 6 independent experiments. \* $P < 0.05$  versus unstimulated control; # $P < 0.05$  versus all other 30' insulin groups; ns, non-significant.



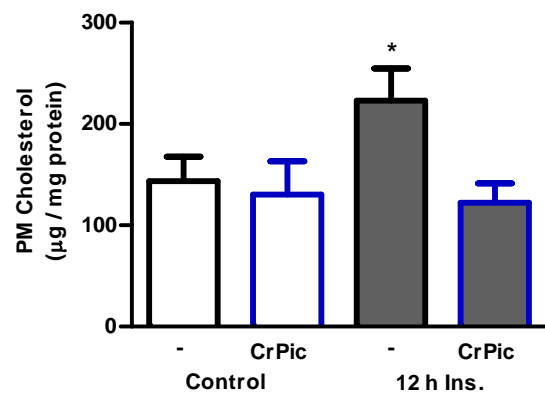
**Figure 2.** CrPic does not enhance nor does hyperinsulinemia impair insulin signaling. After treatments, 3T3-L1 adipocytes were left unstimulated or stimulated by a maximal dose (100 nM) of insulin for 5 minutes to induce phosphorylation of insulin signaling proteins. A, Phosphorylation of Akt2 (Ser 474) normalized to total Akt2. B, Phosphorylation of AS160 normalized to total AS160. Values are mean  $\pm$  SEM from 4 independent experiments. \* $P < 0.05$  versus respective unstimulated cells; ns, non-significant.

### 3.2. CrPic Inhibits PM Cholesterol Accumulation

Studies have demonstrated that hyperinsulinemia promotes increased PM cholesterol, in turn perturbing cortical F-actin necessary for proper GLUT4 incorporation into the PM [8,9]. *Ex vivo* examination of skeletal muscle from insulin-resistant Zucker rats demonstrated that correction of membrane cholesterol restores actin structure and insulin sensitivity [9]. It was examined whether CrPic treatment had beneficial effects on PM cholesterol levels. PM cholesterol content was elevated by approximately 50% in adipocytes exposed to hyperinsulinemia (Figure 3). Importantly, concurrent treatment with CrPic prevented this gain in cholesterol content.

### 3.3. CrPic Prevents O-GlcNAcylation of Sp1

Recent findings have established excessive glucose flux



**Figure 3.** CrPic protects against PM cholesterol accumulation induced by hyperinsulinemia. After treatments, 3T3-L1 adipocytes were lysed and fractionated to prepare purified PM fractions. Cholesterol content in PM fractions was assessed via Amplex Red, normalized to PM protein content. Values are mean  $\pm$  SEM from 5 independent experiments. \* $P < 0.05$  versus all other groups.

through the HBP in provoking a cholesterolgenic response through modification of Sp1 [12]. To test whether CrPic could be potentiating HBP activity, thereby reducing a transcriptional response leading to increased PM cholesterol, the glycosylation status of Sp1 was next examined. These analyses revealed both a gain in global O-GlcNAc levels (data not shown) as well as increased O-GlcNAc of Sp1 induced by hyperinsulinemia (Figure 4). While CrPic treatment did not alter HBP activity in control cells, it blunted the effects of hyperinsulinemia in engaging the HBP.

### 3.4. CrPic Attenuates Sp1 Affinity to and Activity of the HMGR Promoter

Study next sought to analyze whether CrPic may promote optimal glucose transport by inhibiting a HBP-induced cholesterolgenic transcriptional response entailing Sp1. In untreated cells cultured in low or high glucose, a majority of Sp1 is localized to the nucleus [25], although some studies suggest O-GlcNAc mediates its nuclear localization [26,27]. Other data, including our own, suggest that O-GlcNAc modification may serve to increase Sp1 binding to the promoter region of target genes or prevent its degradation [12,28,29]. Chromatin immuno-precipitation revealed a significant increase in Sp1 binding affinity to the HMGR promoter induced by hyperinsulinemia, whereas treatment with CrPic blunted this association (Figure 5). To discern if CrPic could inhibit activation the promoter, plasmids containing the coding sequence (-284 to +36) of HMGR, including 3 Sp1 binding moieties, coupled to luciferase, were electroporated into 3T3-L1 adipocytes (Figure 6(a)). Luciferase activity was elevated approximately 2 fold by hyperinsulinemia (Figure 6(b)). Consistent with CrPic inhibiting this response,



(Figure 7(b)). Together, these data suggest a transcriptional basis for the beneficial effects of CrPic on glucose transport processes.

#### 4. Discussion

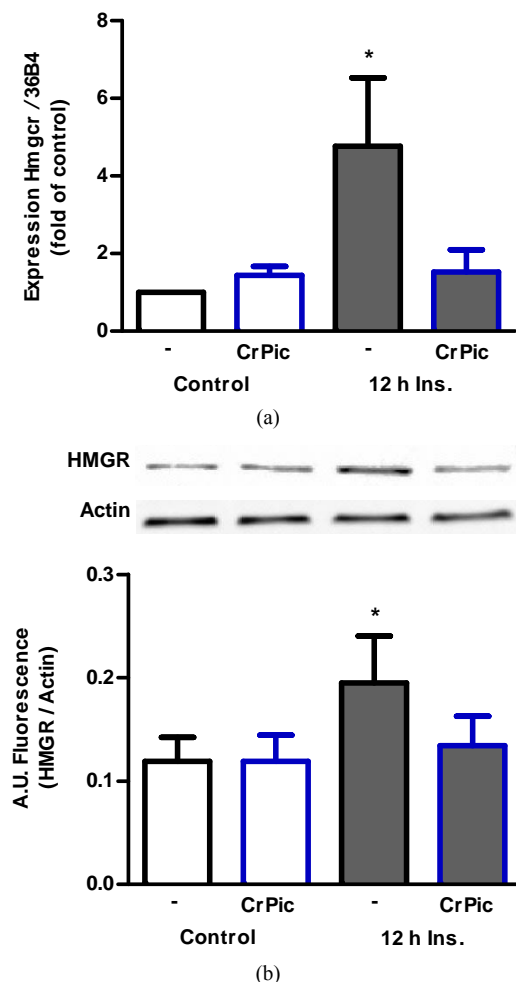
The current studies provide novel mechanistic insight into the established role of CrPic in benefiting glucose homeostasis. Data support a transcriptional basis for CrPic action in preventing PM cholesterol accumulation, which has been previously shown to impinge upon insulin action. While the mechanisms by which CrPic may precisely inhibit glucose flux through and/or activity of the HBP were not a focus of this work, study has demonstrated that CrPic activates AMPK, a known sensor of

low energy status [4,6,13,30,31]. Additionally, it has recently been determined that AMPK can phosphorylate and inhibit glutamine fructose-6-phosphate amidotransferase, the rate limiting enzyme in the HBP [32]. In this regard, CrPic may have pleiotropic effects on cholesterol synthesis processes through inhibiting both production of HMGR as well as its activity.

The exact mechanisms whereby this micronutrient may trigger the activation of AMPK to potentially modulate flux through this pathway are thus warranted and may involve liver kinase B1, calmodulin activated protein kinase kinases, protein tyrosine phosphatase 2A and/or alterations in the cellular ATP/AMP ratio through altering mitochondrial complexes [33-36].

With regard to the HBP, approximately 25% of proteins modified by *O*-GlcNAc are transcription factors [37]. Sp1 was the primary focus of this work due to recent study suggesting inhibition of Sp1 could protect against glucose transport dysfunction in adipocytes [12]. Nevertheless, HBP-induced *O*-GlcNAc on histones has been demonstrated [38]. Interestingly, this study found that many metabolic gene products, including HMGR, were upregulated in response to activation of the HBP. As HBP-induced modification of proteins is known to occur in times of nutrient excess, global inhibition of this pathway by CrPic is of interest as it may have beneficial metabolic effects extending beyond maintenance of cholesterol synthesis. In this regard, a study has shown that CrPic or HBP inhibition restores cholesterol efflux rendered impaired by hyperinsulinemia [13]. While the current study focused on inhibition of cholesterol synthesis, CrPic has been documented to be necessary for maintenance or even improve high density lipoprotein levels in humans given brewer's yeast [39,40]. Together, these findings support a mechanism whereby CrPic may benefit glucose metabolism by maintaining optimal PM cholesterol levels needed for appropriate glucose transport into peripheral tissues.

In terms of human health, our data suggest a novel, putative mechanism whereby CrPic could be beneficial to glucose metabolism through countering PM stress. While clinical trials suggest a beneficial effect of CrPic in diabetics, it may be possible that CrPic treatment has an effect to counter dysregulation in membrane stress and that this incremental effect may become impeded later in disease progression when other factors further perturb insulin sensitivity. Alternatively, antihyperglycemic medications could mask the effects of CrPic, as many are known to activate AMPK. Further, well-designed longitudinal studies are thus needed in insulin-resistant, non-diabetic patients to help better characterize the role of this micronutrient in alleviating insulin resistance. In this regard, CrPic use may prove beneficial in preventing the exacerbation of insulin resistance in patient populations



**Figure 7.** CrPic protects against hyperinsulinemia-induced HMGR synthesis. **A.** After treatments, RNA was purified, reverse transcribed, and HMGR mRNA expression levels were assessed via qPCR and quantitated using the  $\Delta\Delta$ Act method, normalized to 36B4 mRNA expression. **B.** After treatments HMGR protein content was assessed via western blotting, normalized to actin. Values are mean  $\pm$  SEM from 4 - 5 independent experiments. \* $P < 0.05$  versus untreated control.

through promoting optimal PM fluidity.

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