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Docosahexaneoic Acid [22:6,n-3] Regulates Rat Hepatocyte Sterol Regulatory Element Binding Protein-1 (SREBP-1) Nuclear Abundance by Erk- and 26S Proteasome-Dependent Pathways.

Daniela Botolin, Yun Wang, Barbara Christian,
and Donald B. Jump
Departments of Physiology, Biochemistry and Molecular Biology
Michigan State University
East Lansing, Michigan 48824

Corresponding author:
Donald B. Jump, Ph.D.
Department of Physiology
3165 Biomedical and Physical Sciences Building
Michigan State University
East Lansing, MI 48824
Phone: 517-355-6475, ext. 1133
FAX: 517-355-5125
Email: Jump@msu.edu

Abbreviations: BSA, bovine serum albumin; ER, endoplasmic reticulum; LUC, luciferase; LXR, liver X receptor; SREBP, sterol regulatory element binding protein; SCAP, SREBP cleavage activating protein; PUFA, polyunsaturated fatty acid; RT-PCR, real time PCR.

Footnotes:

[1]Unpublished observation.

Abstract: Insulin induces and dietary n-3 polyunsaturated fatty acids (PUFA) suppress hepatic *de novo* lipogenesis by controlling sterol regulatory element binding protein-1 nuclear abundance (nSREBP-1). Our goal was to define mechanisms involved in this regulatory process. Insulin treatment of rat primary hepatocytes rapidly augments nSREBP-1 and $mRNA_{SREBP\text{-}1c}$, while suppressing $mRNA_{Insig\text{-}2}$, but not $mRNA_{Insig\text{-}1}$. These events are preceded by rapid, but transient increases in Akt and Erk phosphorylation. Removal of insulin from hepatocytes leads to a rapid decline in nSREBP-1 ($T_{1/2}$ ~ 10 hrs) that is abrogated by inhibitors of 26S-proteasomal degradation. 22:6,n-3, the major n-3 PUFA accumulating in livers of fish oil-fed rats, suppresses hepatocyte levels of nSREBP-1, $mRNA_{SREBP\text{-}1c}$ and $mRNA_{Insig\text{-}2,}$ but modestly and transiently induces $mRNA_{Insig\text{-}1}$. More importantly, 22:6,n-3 accelerates the disappearance of hepatocyte nSREBP-1 ($T_{1/2}$~ 4 hrs) through a 26S proteasome-dependent process. 22:6,n-3 has minimal effects on microsomal SREBP-1 and SCAP, or nuclear SREBP-2. 22:6,n-3 transiently inhibits insulin-induced Akt phosphorylation, but induces Erk phosphorylation. Inhibitors of Erk phosphorylation, but not overexpressed constitutively active Akt, rapidly attenuate 22:6,n-3 suppression of nSREBP-1. Thus, 22:6,n-3 suppresses hepatocyte SREBP-1 nuclear content through 26S proteasome- and Erk-dependent pathways. These studies reveal a novel mechanism for n-3 PUFA regulation of hepatocyte SREBP-1 nuclear abundance and lipid metabolism.

Introduction.

Sterol regulatory element binding proteins (SREBP-1a, SREBP-1c & SREBP-2) are HLH-bZip transcription factors that play a central role in controlling the transcription of genes involved in cholesterol and fatty acid synthesis (1). The principal mechanism for SREBP regulation of gene transcription involves control of its nuclear abundance (nSREBP). nSREBP is regulated by two post-translational mechanisms, proteolytic processing (1) and 26S proteasomal degradation (2). All SREBPs are synthesized as precursors (pSREBP, ~125 kd) tethered to the endoplasmic reticulum (ER) and escorted to the Golgi complex by SREBP-cleavage activating protein (SCAP) for proteolytic processing. nSREBP is transported to the nucleus, via importin-β (3), where it binds sterol regulatory elements in promoters of specific genes, recruits co-activators to the promoter and stimulates gene transcription (4). Phosphorylation and ubiquitination of nSREBP targets nSREBP for 26S proteasomal degradation (5). Sterols regulate nSREBP levels by controlling the proteolytic processing step, not 26S proteasomal degradation. Instead, sterols induce the ER-resident proteins, Insig-1 and Insig-2 to bind SCAP which retains the SCAP-SREBP complex in the ER, preventing its cleavage to nSREBP (6). This is the molecular basis for cholesterol suppression of nuclear SREBP-2 abundance and the suppression of cholesterol synthesis.

While SREBP-1c and SREBP-2 are structurally similar, their regulation in the liver by nutrients, hormones and during postnatal development is quite different. SREBP-1c, but not SREBP-2, is induced by insulin and liver X receptor (LXR) agonists (7, 8). Both insulin and LXR agonists stimulate *de novo* lipogenesis. Oxysterol-activated LXR/RXR heterodimers bind DR-4 regulatory elements on the SREBP-1c promoter and

induce SREBP-1c gene transcription (8, 9). Insulin induction of SREBP-1c gene transcription has been correlated with PI3-kinase activation and Akt phosphorylation (10-12). Insulin also induces changes in Insig-1, Insig-2a and Insig-2b mRNA abundance (13, 14), implicating effects of insulin on SREBP processing. However, the linkage between the insulin-regulated signaling pathways and the control of SREBP-1c nuclear abundance remains poorly defined.

Polyunsaturated fatty acids (PUFA) suppress the nuclear abundance of SREBP-1 (nSREBP-1), but not nSREBP-2. The decline in nSREBP-1 accounts for the PUFA-mediated suppression of *de novo* lipogenesis (15, 16). The mechanism for the suppression of nSREBP-1 is complex and has been attributed to inhibition of SREBP-1c gene transcription, enhanced $mRNA_{SREBP\text{-}1}$ degradation as well as inhibition of SREBP-1 proteolytic processing (16-25). In some established cell lines, PUFA interfere with oxysterol-activated LXRα receptors (26, 27). However, in primary hepatocytes and rat liver, PUFA do not interfere with LXR-regulated gene expression (28).

Our goal is to identify pathways regulated by insulin and n-3 PUFA that control SREBP-1 nuclear content (nSREBP-1). We will show that insulin and n-3 PUFA rapidly control SREBP-1 nuclear abundance through post-translational mechanisms. Moreover, both insulin and n-3 PUFA affect the phosphorylation status of Akt and Erk which impacts SREBP-1c nuclear abundance. Our studies reveal a previously unrecognized mechanism by which n-3 PUFA control SREBP-1 nuclear abundance.

Methods and Materials.

***Animals and Primary Hepatocytes*.** All procedures for the use and care of animals for laboratory research have been approved by the All University Committee for Animal Use and Care at Michigan State University.

Feeding study: Male Spraque-Dawley rats were acclimated to meal-feeding a high carbohydrate (glucose) diet (HiCHO) [ICN Biochemicals, Aurora, OH] supplemented with olive oil (Pompiean, Baltimore, MD) at 10% w/w for 7 days. The meal began at 8 AM and ended at noon. After the acclimation period, rats were either maintained on the olive oil diet or switched to a HiCHO diet supplemented with fish oil (Dyets, Inc., Bethlehem, PA) at 10% w/w (29, 30). Animals were maintained on the olive oil or fish oil for 7 days. Two hours after completion of the final meal animals were euthanized for tissue collection.

Primary Hepatocytes: Rats are maintained on Harlan-Teklad laboratory chow (#8640) and water *ad lib*. Rat primary hepatocytes were prepared from Teklad chow-fed (*ad lib*) male Spraque-Dawley rats, cultured on BioCoat (type 1 collagen) plates (Beckon Dickinson, Belford, MA.) and treated with insulin (Invitrogen, Carlsbad, CA) or fatty acids (Nu-Chek Prep, Elysian, MN) as previously described (28)

Quantitation of hepatic fatty acids composition. Total lipid was extracted from liver in chloroform:methanol (2:1) plus 1 mM butylated hydroxytoluene (30). 7-nonadecenoic acid (19:1) was added as a recovery standard at the time of extraction. Protein (Bio-Rad, Hercules, CA) was measured in extracts after the initial homogenization step. Total lipids were saponified, fractionated and quantified by reverse phase HPLC (RP-

HPLC) using a YMC J-Sphere (ODS-H80) column and a sigmoidal gradient starting at 86.5% acetonitrile + acetic acid (0.1%) and ending at 100 % acetonitrile + acetic acid (0.1%) over 50 mins with a flow rate of 1.0 ml/min using a Waters 600 controller. Fatty acids were detected using both UV absorbance at 192 nm (Waters model 2487) and evaporative light scatter (Waters model 2420). Fatty acid composition and structures were confirmed at the MSU Mass Spectrometry facility by GC/MS (www.bch.msu.edu/facilities/massspec/index.html). Fatty acid standards for RP-HPLC were obtained from Nu-Chek Prep (Elysian, MN).

RNA extraction and real time-PCR. RNA was extracted from primary hepatocytes (30) and used as template for real time-PCR. Specific primers for each gene (see below) were designed using Primer Express software (Applied Biosystems, Foster City, CA). First strand cDNA was synthesized using the SuperScript II RNase H- Reverse Transcriptase (Invitrogen Carlsbad, CA). Synthesized cDNA was mixed with 2x SYBR Green PCR Master Mix (Applied Biosystems) and various sets of gene-specific forward and reverse primers and subjected to real-time PCR quantification using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. The relative amounts of mRNAs were calculated by using the comparative C_T method. Cyclophilin was used as a control and all results were normalized to the abundance of cyclophilin mRNA. Primers used for real time PCR:

SREBP-1c Forward Primer: TGGACTACTAGTGTTGGCCTGCTT

Reverse Primer: ATCCAGGTCAGCTTGTTTGCGATG

Insig-1	Forward Primer:	TGCAGATCCAGCGGAATGT
	Reverse Primer:	CCAGGCGGAGGAGAAGATG
Insig-2a	Forward Primer:	GACGGATGTGTTGAAGGATTTCT
	Reverse Primer:	TGGACTGAAGCAGACCAATGTC
Insig-2b.	Forward Primer:	CCGGCAGAGCTCAGGATTT
	Reverse Primer:	AACTGTGGACTGAAGCAGACCAA
Cyclophilin.	Forward Primer:	TGGATGGCAAGCATGTGGTCTTTG
	Reverse Primer:	CTTCTTGCTGGTCTTGCCATTCCT.

Immunoblotting. Extracts of primary hepatocytes were prepared as described previously (30, 31). Proteins (50-100 μg) extracted from cytosolic, microsomal, and nuclear fractions were separated electrophoretically by SDS-polyacrylamide gel electrophoresis (NuPAGE 4-12% polyacrylamide Bis-Tris, Invitrogen) and transferred to nitrocellulose membranes. Membranes were incubated with antibodies for SREBP-1 (IgG-2A4, sc-13551), SREBP-2 (IgG-7D4, sc-5603), SCAP (sc-9675), Akt1 (sc-1618), pAkt1/2/3-Ser^{473} (sc-7985), ERK1 (sc-94), phosphor-ERK (sc-7383) (Santa Cruz Biotechnology, San Cruz, CA). Anti-mouse and anti-rabbit secondary antibodies were obtained from Bio-Rad (Hercules,CA); anti-goat antibodies were obtained from Santa Cruz Biotechnology. The SuperSignal West Pico chemiluminescence kit (Pierce) detection system was used.

Recombinant Adenovirus Infection—Recombinant adenovirus expressing luciferase, kinase-dead-Akt (Adv-Akt-KD) and constitutively active-Akt (Adv-Akt-CA) were obtained from C. Rhodes, Pacific Northwest Research Institute, Seattle, WA (32). Confluent

primary hepatocytes were infected (10 plaque forming units/cell) and harvested for evaluation of microsomal and nuclear SREBP-1 abundance.

***Statistical Analysis*.** Statistical analysis used Student's t-test and ANOVA plus post hoc Tukey HSD test (http://faculty.vassar.edu/lowry/VassarStats.html).

Results.

Effect of dietary fat on hepatic lipid composition. When compared to olive oil-fed rats, rats fed n-3 PUFA (fish oil)-enriched diets have lower levels of nSREBP-1 and SREBP-1 target genes (28). The mole % of the n-3 PUFA in the fish oil diet is: 18:3,n-3 (1.8%), 20:5,n-3 (13%), 22:5,n-3 (1.7%) and 22:6,n-3 (16.3%). Analysis of the hepatic fatty acid composition of animals fed these diets indicates that fish oil feeding leads to no significant change in the total amount of lipid in the liver, but significant changes in the type of fatty acids (Table 1). Despite the nearly equal level of 20:5,n-3 and 22:6,n-3 in the diet, 22:6,n-3 accumulates in livers of fish oil-fed rats. Other n-3 PUFA in the fish oil diet, i.e., 18:3 n-3, 20:5,n-3 and 22:5 n-3, are likely elongated, desaturated and β-oxidized in the peroxisome to form 22:6,n-3, the end product of n-3 PUFA synthesis (33). Hepatic enrichment of 22:6,n-3 occurs in neutral lipids (triglycerides and cholesterol esters), phospholipids and non-esterified fatty acids (not shown).

Differential effects of n-3 PUFA on rat hepatic SREBP-1 regulation. Since 22:6,n-3 accumulate in livers of fish oil-fed rats, we examined the effect of 22:6,n-3 on SREBP-1 precursor and nuclear abundance in primary hepatocytes. Increasing the dose of 22:6,n-3 to 250 μM suppressed both precursor and nuclear forms of SREBP-1. However, SREBP-1 nuclear (nSREBP-1) abundance was more sensitive to 22:6,n-3 suppression than microsomal SREBP-1, pSREBP-1 (Fig. 1A). We next compared the effect of 20:5,n-3 and 22:6,n-3 on SREBP-1 abundance. At the100 μM dose both fatty acids suppressed microsomal pSREBP-1 equally, 25-40% (Fig. 1B & C). 20:5,n-3 and 22:6,n-3 suppressed SREBP-1 nuclear content by 43 and 76%, respectively. 22:6,n-3 was ~2-fold more effective than 20:5,n-3 at suppressing nSREBP-1.

Rapid effects of insulin and 22:6,n-3 on hepatic nSREBP-1c. Since 22:6,n-3 accumulates in livers of fish oil fed rats (Table 1) and is more potent than 20:5,n-3 at suppressing nSREBP-1 (Fig. 1), 22:6,n-3 was used to examine the time course of n-3 PUFA effects on the regulation of nSREBP-1, pSREBP-1 and $mRNA_{SREBP\text{-}1c}$ in rat primary hepatocytes. Cells incubated overnight in serum-free Williams E medium containing no insulin lowered nSREBP-1 by ~80% (Fig. 2A), with minimal (~10%) effect on microsomal pSREBP-1 abundance (Fig. 2B). Addition of insulin (10 nM) to the culture medium induced nuclear SREBP-1, SREBP-1 precursor and $mRNA_{SREBP\text{-}1c}$ (Fig. 2 A-C) 6-, 1.2- and 5-fold after 24 hrs. 22:6,n-3 rapidly and significantly attenuated the insulin induction of SREBP-1 nuclear protein (Fig. 2A) but modestly suppressed microsomal SREBP-1 (Fig. 2B). 22:6,n-3 had no effect on SREBP-2 nuclear abundance (Fig. 2A insert).

Both 20:5,n-3 and 22:6,n-3 suppressed the insulin induction of $mRNA_{SREBP\text{-}1c}$. While 20:5,n-3 effects on $mRNA_{SREBP\text{-}1c}$ appear transient, 22:6,n-3 has a sustained suppressive effect on $mRNA_{SREBP\text{-}1c}$. The fact that 22:6,n-3 also lowered the abundance of $mRNA_{SREBP\text{-}1c}$ by ~70% over 24 hr implicates enhanced mRNA decay. 20:5,n-3 also promoted a decline in $mRNA_{SREBP\text{-}1c}$ at 6 hrs, but by 24 hrs $mRNA_{SREBP\text{-}1c}$ levels increased. Thus, 22:6,n-3 has a sustained suppressive effect on $mRNA_{SREBP\text{-}1c}$, while the 20:5,n-3 effect on $mRNA_{SREBP\text{-}1c}$ is transient.

Taken together, these studies indicate that insulin rapidly induces SREBP-1 nuclear abundance and that 22:6,n-3 rapidly inhibits this response (Figure 2). Neither factor had a major effect on microsomal SREBP-1 (Fig. 2B) or nuclear SREBP-2 (Fig. 2A). Although both insulin and 22:6,n-3 affected $mRNA_{SREBP\text{-}1c}$ (Figure 2C), the absence of a parallel change in microsomal SREBP-1 suggest that both insulin and 22:6,n-3 act rapidly at a post-translational level to control SREBP-1 nuclear abundance.

Effects insulin and 22:6,n-3 on SCAP, Insig-1 and Insig-2. SREBP nuclear abundance is regulated by two opposing post-translational mechanisms, SREBP processing (1) and 26 S proteasomal degradation(2). We examined the effect of 22:6,n-3 on components involved in SREBP processing, e.g., SCAP, Insig-1 and Insig-2.

Microsomal SCAP levels remained marginally affected by insulin or n-3 PUFA (Fig. 3A). Insig-1 mRNA abundance was modestly increased by insulin. 22:6,n-3 transiently induced Insig-1 mRNA in the presence and absence of insulin (Figure 3B). Insig-2A mRNA was rapidly suppressed by insulin and 22:6,n-3 (Figure 3C). Insig-2B levels were unaffected by insulin or 22:6,n-3 (not shown).

The effects of insulin on Insig-1 and Insig-2 mRNA abundance confirm earlier reports (13). 22:6,n-3 effects on Insig-1 and Insig-2 expression suggest that n-3 PUFA change Insig subtype expression in the liver. However, the effect of 22:6,n-3 + insulin on Insig-1 expression is modest and transient and may be insufficient to impact SREBP-1 processing. Finding that both insulin and 22:6,n-3 suppressed Insig-2 mRNA abundance is inconsistent with a role of Insig-2 in the 22:6,n-3 regulation of SREBP-1 nuclear abundance. The outcome of these studies suggest that 22:6,n-3 may have minimal effects on SREBP-1 processing.

Effect of 26S proteasome inhibitors on insulin and 22:6,n-3 control of SREBP-1 nuclear abundance. SREBP-1 and -2 are ubiquitinated and degraded by a 26S proteasome-dependent pathway (2, 5). To determine if either insulin or 22:6,n-3 affect SREBP-1 26S proteasomal degradation, primary hepatocytes incubated overnight in serum- and insulin-free medium were treated with insulin or insulin + 22:6,n-3 in the absence and presence of 26S proteasome inhibitors, MG132 or lactacystin (Figure 4A).

Lactacystin had no effect on basal or insulin induced (4-fold) SREBP-1 nuclear abundance (Fig. 4A, insert). Treatment of hepatocytes with insulin + 22:6,n-3 significantly (>90%) suppressed nSREBP-1. Co-incubation with lactacystin or MG132 significantly attenuated the 22:6,n-3 effect on nSREBP-1 abundance (Figure 4A). Incubating primary hepatocytes with 22:6,n-3 alone reduced SREBP-1 to undetectable levels; inclusion of lactacystin restored SREBP-1 to a detectable level. These results suggest that 22:6,n-3 regulates SREBP-1 nuclear abundance through a 26S-proteasome-dependent process.

To examine this process differently, primary hepatocytes were treated with insulin overnight to induce nSREBP-1. Cells were maintained for an additional 24 hrs with insulin or the insulin was withdrawn; 22:6,n-3 was added to one group of cells in which insulin was withdrawn (Figure 4B). Maintaining insulin treatment for an additional 24 hrs had little effect on SREBP-1 nuclear abundance. However, removal of insulin leads to a rapid decline in SREBP-1 from the nucleus, ~$T_{1/2}$ = 10 hrs. Addition of 22:6,n-3 to the minus-insulin group accelerates the loss of nSREBP-1, ~$T_{1/2} \leq$ 4 hrs.

This study was repeated with the addition of MG132 and the cells were harvested 12 hr after insulin removal (Fig. 4C). Removal of insulin resulted in ~80% decrease in nSREBP-1 after 12 hrs; addition of 22:6,n-3 suppressed nSREBP-1 to 96% loss. Addition of MG132 totally prevented the loss of nSREBP-1 in cells receiving no insulin in the absence and presence of 22:6,n-3.

Lactacystin (Figure 4D) or MG132 (not shown) had no consistent effect on SREBP-1 precursor or nuclear nSREBP-2 abundance. Moreover, suppression of SREBP-1 nuclear abundance by 20:5,n-3 and 22:5,n-3 was not affected by lactacystin. Thus, the action of 26S proteasome inhibitors (lactacystin & MG132) on SREBP-1 nuclear abundance is specific for 22:6,n-3. The outcome of these results support the notion that 22:6,n-3 regulates SREBP-1 nuclear abundance through a 26S proteasome-dependent mechanism.

Akt is a target for both insulin and 22:6,n-3 regulation. In this section, we focused on determining which signal transduction mechanisms were involved in the regulation of SREBP-1 nuclear abundance. Insulin activation of PI3 kinase and Akt are established

routes for insulin control of nSREBP-1 (10, 12). We confirmed the involvement of PI3 kinase by using the inhibitor, LY294002, to block the insulin-mediated induction of SREBP-1 nuclear abundance in primary rat hepatocytes (not shown). The effect of insulin and 22:6,n-3 on Akt phosphorylation (Ser^{473}) was examined in primary hepatocytes (Fig. 5A).

Removal of insulin from the medium overnight lowers Akt-phosphorylation, while insulin addition induces hepatocyte Akt-phosphorylation 8-fold within 1.5 hrs. Six hours after insulin treatment, Akt-phosphorylation was ~2-fold above basal values. 22:6,n-3 significantly attenuated (50%) the insulin-stimulated Akt phosphorylation at 1.5 hrs, but had no significant effect on Akt phosphorylation after 6 hrs. The immunoblot (Fig. 5A, insert) illustrates the effect of insulin, 22:6,n-3 and the combination treatment on Akt phosphorylation at the 1.5 hr. time point. Total Akt protein levels remained unaffected by insulin or 22:6,n-3 treatment. Note that cells treated with 22:6,n-3 alone had no effect on Akt phosphorylation. Thus, 22:6,n-3 only affects insulin-stimulated Akt phosphorylation.

Since insulin-induced Akt phosphorylation was attenuated by 22:6,n-3, we determined if over expression of a constitutively active form of Akt would affect the 22:6,n-3 control of nSREBP-1 in primary hepatocytes. In a preliminary study, primary hepatocytes were infected with recombinant adenovirus expressing luciferase (Ad-Luc, as a control), Akt-kinase dead form (Adv-Akt-KD) or constitutively active Akt (Adv-Akt-CA) (Fig. 5B). The conditions of infection resulted in $\geq$10-fold increase in Akt-CA and Akt-KD expression (not shown). Cells received no insulin treatment. When compared to uninfected cells, Adv-Luc and Akt-KD had little affected on microsomal SREBP-1 (pSREBP-1). Akt-KD suppressed nSREBP-1 by ~50%. Akt-CA significantly induced

SREBP-1c nuclear form (3-fold) with little effect on SREBP-1 precursor. Thus, changes in Akt activity affect SREBP-1 nuclear abundance through a post-translational mechanism.

To determine if over expressed Akt (Adv-Akt-CA) could abrogate 22:6,n-3 control of SREBP-1, primary hepatocytes were infected with Ad-Luc and Adv-Akt-CA and treated with insulin, 22:6,n-3 or insulin + 22:6,n-3 for 6 hrs (Fig. 5C & D). Microsomal pSREBP-1 remained unaffected by insulin or 22:6,n-3 in Ad-Luc or Adv-Akt-CA infected hepatocytes (Fig. 5C). Adv-Akt-CA infection induced nSREBP-1 nearly 3-fold and insulin treatment of both Ad-Luc and Adv-Akt-CA had modest effects on nSREBP-1. More importantly, 22:6,n-3 treatment of hepatocytes, in the absence or presence of insulin, significantly suppressed nSREBP-1 in both Ad-Luc and Adv-Akt-CA treated cells. Overexpressed Akt-CA failed to abrogate the 22:6,n-3 effect on nSREBP-1. Thus, the inhibitory effect of 22:6,n-3 on Akt phosphorylation (Fig. 5A) is not linked to the 22:6,n-3-mediated suppression of nSREBP-1 (Fig. 5D).

Effects of insulin and 22:6,n-3 on Erk phosphorylation. Insulin regulation of MAP-kinase signaling pathways is well established (14, 34). Insulin treatment of primary rat hepatocytes rapidly, but transiently, increased Erk phosphorylation (Fig. 6A). This biphasic response has been described by other investigators (14). The combination of 22:6,n-3 and insulin had no effect on the early induction of Erk phosphorylation suggesting that 22:6,n-3 does not interfere with insulin simulated Erk phosphorylation. However, 24 hrs after initiating treatment, Erk phosphorylation was sustained at a level significantly above the insulin treated group. Insulin and 22:6,n-3 had no effect on total

Erk protein abundance. Treatment of primary hepatocytes with 22:6,n-3 alone also increased Erk phosphorylation after 3 hrs (not shown). Repeating this study with palmitate, 20:5,n-3 or 22:6,n-3 (Fig. 6B) indicates that after 24 hrs of treatment, only 22:6,n-3 significantly induced Erk phosphorylation. Elevated Erk phosphorylation correlates with robust suppression of SREBP-1 nuclear content.

To determine if Erk phosphorylation is linked to the regulation of SREBP-1 nuclear content, cells were treated overnight with 10 nM insulin in the absence and presence of 100 μM 22:6,n-3 (Fig. 7). Overnight insulin treatment induced nSREBP-1, but had no effect on Erk phosphorylation. Overnight treatment with insulin + 22:6,n-3 suppressed nSREBP-1 and induced Erk phosphorylation (see immunoblot and graph). The two groups of hepatocytes were treated with vehicle or MEK inhibitors of Erk phosphorylation, 10 nM PD98059 or 10 nM U0126. After 30 minutes of treatment, hepatocytes were harvested for analysis of SREBP-1 nuclear content and Erk phosphorylation. The results using PD98059 are quantified in Fig. 7 (Graph). Treatment of primary hepatocytes with PD98059 or U0106 effectively suppressed 22:6-induced Erk phosphorylation. More surprisingly, these MEK inhibitors (PD98059 and U0106) significantly induced SREBP-1 nuclear content in cells treated with insulin + 22:6,n-3 overnight. These results indicate that chronic suppression of nSREBP-1 by 22:6,n-3 is rapidly (within 30 mins) reversed by treatment with MEK inhibitors (PD98059 or U0106) of Erk phosphorylation.

The p38 inhibitor, SB203580, had no effect on Erk phosphorylation or SREBP-1 nuclear content. The Jnk inhibitor, SP600125, was found unsuitable for our studies

because it stimulated Erk phosphorylation. Taken together, these results indicated that 22:6,n-3 regulates SREBP-1 nuclear abundance through an Erk-dependent mechanism.

Discussion.

Insulin and n-3 PUFA regulate hepatic *de novo* lipogenesis, at least in part, by controlling SREBP-1 nuclear abundance (1, 16, 35). Our goal was to define mechanisms involved in this regulatory process. These studies have revealed a novel pathway by which one n-3 PUFA, namely 22:6,n-3, rapidly controls SREBP-1 nuclear abundance. The evidence presented in this report supports the concept that 22:6,n-3 accelerates the degradation of nuclear SREBP-1 abundance by a 26S proteasome-dependent pathway while having little impact on microsomal SREBP-1 or nuclear SREBP-2 (Figs. 1 - 4). 22:6,n-3 is the major n-3 PUFA accumulating in livers of fish oil-fed animals (Table 1). Moreover, 22:6,n-3 is the end product of n-3 PUFA synthesis from the essential fatty acid, 18:3 n-3 (33). As such, 22:6,n-3 is a feedback inhibitor of its own synthesis. Fish oil feeding suppresses the expression of key hepatic enzymes in this pathway, specifically Elovl-5, Δ^5 and Δ^6 desaturase and activates peroxisomal β-oxidation for the degradation of $\geq$C22 PUFA through a PPARα-dependent mechanism (29, 36) This regulatory scheme is analogous to the cholesterol control of its own synthesis by SREBP-2 (1), but with an important distinction. Cholesterol regulates the proteolytic conversion of SREBP-2 precursor to the nuclear form with little impact on 26S proteasomal degradation or SREBP-2 gene expression (1, 5). In contrast, 22:6,n-3 suppresses SREBP-1 nuclear abundance by accelerating 26S proteasome-dependent degradation of nSREBP-1 (Fig. 4). Like 20:5,n-3, 22:6,n-3 also controls SREBP-1

nuclear abundance through effects on SREBP-1c promoter activity[1] and $mRNA_{SREBP-1}$ abundance (Fig. 2C). However, 22:6,n-3 is the only n-3 PUFA controlling SREBP-1c nuclear abundance through a 26S proteasome dependent mechanism (Fig. 4D).

SREBP-1a, -1c and -2 are ubiquitinated and degraded by the 26S proteasome (2, 5, 37). The signal initiating SREBP degradation involves phosphorylation of ^{426}Thr and Ser^{430} in the ^{424}TLTTPPPSD motif in SREBP-1a and ^{432}Thr and ^{436}Ser in the ^{430}LMSPPASD motif of SREBP-2 (2). These sites correspond to ^{393}Thr and ^{399}Ser in SREBP-1c. Phosphorylation of the TLTTPPPSD motif by Gsk3β promotes binding of the ubiquitin ligase, SCF^{Fbw7}, which targets SREBP for 26S proteasomal degradation. Inhibition of Gsk3 activity by LiCl (38) or insulin (2) promotes the accumulation of SREBP. Insulin inhibits Gsk3β activity by increasing the Akt-mediated phosphorylation of Gsk3β at Ser^{9}.

Our studies support the concept that insulin rapidly controls SREBP-1 nuclear content by regulating SREBP-1 degradation. Over expression of kinase-dead Akt suppresses nSREBP-1, while constitutively active Akt induces nSREBP-1 in the absence of insulin. Over expression of these Akt variants have little effect on microsomal SREBP-1 abundance (Fig. 5B). Moreover, insulin induction of Akt phosphorylation (Fig. 5A) precedes the elevation in SREBP-1 nuclear abundance with minimal effects on SREBP-1 precursor (Fig. 2). Removal of insulin from hepatocytes promotes a rapid decline in nSREBP-1 ($T_{1/2}$ ~10 hr) (Fig. 4B) that is abrogated by 26S proteasome inhibitors (Fig. 4C). Taken together, these results suggest that insulin acts rapidly to induce SREBP-1 nuclear abundance by inhibiting 26S proteasomal degradation. Insulin effects on SREBP-1 promoter activity (22), $mRNA_{SREBP-1}$ and

microsomal SREBP-1 abundance (Fig. 2) may be secondary to the primary effects of insulin on SREBP-1 phosphorylation and its proteasomal degradation.

22:6,n-3 suppresses insulin-induced SREBP-1 nuclear content with little impact on microsomal SREBP-1 or nuclear SREBP-2 (Fig. 1, 2 & 4D). The transient inhibition of 22:6,n-3 on insulin-induced Akt phosphorylation (Fig. 5A) might be expected to enhance Gsk3β-mediated phosphorylation of SREBP-1 and promote SREBP-1 degradation in the proteasome. However, overexpression of constitutively-active Akt failed to abrogate 22:6-mediated suppression of nSREBP-1 (Fig. 5C & D). Thus, 22:6,n-3 suppression of Akt phosphorylation is not the sole route for 22:6,n3 control of SREBP-1c nuclear abundance.

Insulin also rapidly, but transiently, induces Erk phosphorylation (Fig. 6) (14). Like Akt phosphorylation, insulin induction of Erk phosphorylation precedes changes in nSREBP-1 (Figs. 2 & 6). 22:6,n-3 does not impair the early phase of insulin induced Erk phosphorylation, but sustains elevated Erk phosphorylation after 6 hours (Fig. 6A). Enhanced Erk phosphorylation correlates with lower levels of nuclear SREBP-1 content in hepatocytes (Fig. 6) and in liver[1]. MEK inhibitors (PD95098 and U0126) rapidly (within 30 minutes) attenuate Erk phosphorylation and induce SREBP-1c nuclear abundance (Fig. 7). The rapidity of the MEK inhibitors on SREBP-1 nuclear abundance supports a role of the MEK/Erk pathway in controlling SREBP-1c nuclear abundance.

SREBP-1a is phosphorylated by Erk *in vitro* at Ser^{117} and mutation of this site impairs insulin-stimulated regulation of the LDLR promoter by SREBP-1a (39). Ser^{117}corresponds to Ser^{92} in SREBP-1c. Other Erk sites have been identified in SREBP-1c at Ser^{39}, Ser^{73}, and Thr^{395} (http://scansite.mit.edu/). Based on *in silica*

analysis, Thr^{395}, which is located within the ubiquitin ligase binding motif of SREBP-1 (TLTTPPPSD), may be phosphorylated by both Gsk3β and Erk. Erk phosphorylation of this site may induce ubiquitin ligase binding and target SREBP-1 to the 26S proteasome. Interestingly, ^{430}LMSPPASD in SREBP-2 is also phosphorylated by Gsk3β and binds SCF^{Fbw7} (2), but is not a Erk phosphorylation site (www.scansite.mit.edu). This sequence difference may account for the selective effect of 22:6,n-3 on SREBP-1 26S proteasomal degradation. Clearly, more studies will be required to evaluate the role the ^{393}TLTPPPSD motif plays in 22:6-mediated control of 26S proteasomal degradation of SREBP-1.

An important outcome of our study was finding that not all n-3 PUFA regulate SREBP-1 nuclear content at the level of the 26S proteasome. 18:3 n-3 is a weak regulator of $mRNA_{SREBP-1}$ and nSREBP-1 (not shown). While both 20:5,n-3 and 22:6,n-3 suppress $mRNA_{SREBP-1}$, the 20:5,n-3 effect is transient (Fig. 2C). Both 20:5,n-3 (21) and 22:6,n-3[1] suppress SREBP-1c promoter activity. Thus, the decline in $mRNA_{SREBP-1}$ is likely due to inhibition of SREBP-1c gene transcription and enhanced $mRNA_{SREBP-1}$ decay. The SREBP-1c promoter contains an SRE and SREBP-1c promoter activity is induced by elevated nSREBP-1 (8, 9). We suggest that 22:6-mediated activation of 26S proteasomal degradation of nuclear SREBP-1 may be an antecedent mechanism for 22:6,n-3 control of SREBP-1c gene transcription. However, 26S proteasomal degradation of nSREBP-1 can not explain the n-3 PUFA control of $mRNA_{SREBP-1}$ degradation. PUFA-regulated signaling pathways controlling $mRNA_{SREBP-1}$ degradation remain unresolved.

Inhibitors of 26S proteasomal regulation are able to abrogate much, but not all of the suppressive effect of 22:6,n-3 effect on SREBP-1c nuclear abundance (Figs. 4A & C). Effects of 22:6,n-3 effects on $mRNA_{SREBP-1}$ abundance as well as the modest effects on $mRNA_{Insig-1}$ and microsomal SCAP levels (Fig. 3) may represent additional mechanisms contributing to the 22:6,n-3 control of SREBP-1 nuclear abundance (Fig. 4). Unlike cholesterol control of SREBP-2 (1), 22:6,n-3 acts at multiple levels to control SREBP-1 nuclear abundance.

In summary, our studies have identified a novel mechanism by which n-3 PUFA suppresses nuclear levels of SREBP-1 in rat primary hepatocytes. 22:6,n-3, the main n-3 PUFA accumulating in livers of fish oil-fed rats, accelerates the loss of SREBP-1 from hepatocyte nuclei through 26S proteasome- and Erk-dependent pathways. Inhibition of these pathways abrogates the 22:6,n-3 effect on nuclear SREBP-1 abundance. The outcome of these studies has generated new unanswered questions. In particular, the mechanism for 22:6,n-3 control of MEK activity, Erk phosphorylation and 26S proteasomal degradation of nSREBP-1 remain unresolved. Because insulin and 22:6,n-3 acts rapidly to control SREBP-1 nuclear abundance, it will be important to determine if the effects of insulin and n-3 PUFA on SREBP-1c promoter activity (21, 25) are due to antecedent post-translational mechanisms controlling SREBP-1 nuclear abundance.

Tables

Table 1. Effect of olive and fish oil feeding on hepatic fatty acid composition.

Fatty Acid	Olive Oil	Fish Oil	Mass Change	P-Value
	nmoles/mg protein			
16:0	527 ± 218	391 ± 128	-136	0.21
18:0	55 ± 24	19 ± 13	-36	0.041*
18:1,n-9	224 ± 95	60 ± 18	-164	0.021*
18:2,n-6	127 ± 45	66 ± 2	-61	0.05*
18:3,n-3	8 ± 5	16 ± 2	+8	0.026*
18:3,n-6	9 ± 5	5 ± 1	-4	0.17
20:3,n-6	12 ± 8	3 ± 0.3	-9	0.047
20:3,n-9	26 ± 13	0.5 ± 0.1	-25.5	0.012*
20:4,n-6	204 ± 106	115 ± 15	-89	0.11
20:5,n-3	5 ± 3	75 ± 15	+70	0.0007*
22:5,n-3	5 ± 3	25 ± 8	+20	0.009*
22:6,n-3	236 ± 129	568 ± 150	+332	0.022*
Total	1437 + 621	1341 + 333	-96	0.414
	% of Total			
18:3,n-3	0.6	1.2		
20:5,n-3	0.4	5.6		
22:5,n-3	0.4	1.9		
22:6,n-3	16.4	42.4		

Male Spraque-Dawley rats were meal fed an olive or fish oil-supplement high carbohydrate diet for 7 days (Materials and Methods). The meal consisted of a high carbohydrate diet supplemented with either olive or fish oil at 10% (w/w) (28). Total hepatic lipid was extracted, saponified and quantified (Materials and Methods). Results were obtained from 3 separate animals/group; mean ± S.D. Statistical analysis used the Students t-test; one-tailed P-values were calculated (http://faculty.vassar.edu/lowry/VassarStats.html). *P-values ≤ 0.05 reflect significant differences between the means of the olive and fish oil-fed groups.

Figure Legends.

Figure 1. Effects of n-3 PUFA on SREBP-1 abundance in primary hepatocytes. Primary hepatocytes were incubated overnight in Williams E + 20 mM lactate + 10 nM DEX with no insulin or serum. The next day, cells were treated with 10 nM insulin, 25 mM glucose in the absence and presence of n-3 PUFA with BSA (fatty acid:BSA = 5). **A:** Primary hepatocytes were treated without and with varying concentrations of 22:6,n-3. Cells were harvested after 24 hrs for isolation of microsomal and nuclear proteins for measurement of precursor (pSREBP-1, solid line) and nuclear SREBP-1 (nSREBP-1, dashed line) by immunoblotting (Materials and Methods). The antibody recognizes both SREBP-1a and SREBP-1c. Results are presented as "% of Control" following treatment with fatty acids. Results are representative of 2 separate experiments. **B:** Cells were treated without or with 100 μM 20:5,n-3 or 22:6,n-3 for 24 hrs. Microsomal and nuclear protein was extracted for measurement of precursor (pSREBP-1) and nuclear (nSREBP-1) by immunoblotting (Methods and Materials). **C:** Results of 5 separate experiments were quantified, presented as Arbitrary Density Units (Mean ± S.D.) and evaluated using ANOVA plus post hoc Tukey HSD test (http://faculty.vassar.edu/lowry/VassarStats.html). * $p<0.05$. Veh vs 22:6,n-3.

Figure 2. Time Course of insulin and 22:6,n-3 regulation of microsomal and nuclear SREBP-1 abundance in rat primary hepatocytes. Primary rat hepatocytes were maintained overnight in Williams E medium containing + 20 mM lactate + 10 nM DEX with no serum or insulin. The next morning cells were switched to medium

supplemented with 25 mM glucose and 10 nM insulin in the absence and presence of 100 μM 22:6,n-3. Cells were harvested at the times indicated for extraction of nuclear (**A**) and microsomal (**B**) proteins for measurement of SREBP-1 by immunoblotting. Inserts in A and B are representative immunoblots of SREBP-1 and SREBP-2 after 24 hrs of treatment. Immunoblots (A and B) were quantified and the results are normalized to the level of SREBP-1 expressed in livers of 90 day old male rats, mean ± SD, N=3. **(C)** Primary hepatocytes were incubated overnight as described above. The next day cells were switched to medium supplemented with 25 mM glucose and 10 nM insulin in the absence or presence of 20:5,n-3 or 22:6,n-3 at 100 μM. At the time indicated, cells were harvested for RNA extraction and measurement of SREBP-1c and cyclophilin mRNA by RT-PCR (Methods and Materials). Results are expressed as Fold Change in SREBP1c/Cyclophilin, mean ± SD, N=6.

Figure 3. Effects of n-3 PUFA on SCAP, Insig-1 and Insig-2 expression.

A. Primary rat hepatocytes were treated without and with insulin and 20:5,n-3 or 22:6,n-3 for 24 hrs. After treatment, microsomal protein was isolated for analysis of SCAP protein by immunoblot. The level of SCAP protein was quantified and the results represented in the insert as % Control. Results are representative of 2 separate studies. **B and C**. Total RNA was extracted from primary hepatocytes treated as described in Figure 2C. Levels of Insig-1 (**B**), Insig-2 (**C**) and cyclophilin mRNA were measured by RT-PCR. Results are presented as Fold Change in Insig/Cyclophilin and are representative of 3 separate studies with triplicate samples/treatment. Mean ± SD.

Insulin treated cells, solid line-circles; 22:6,n-3 treated cells, dashed line, squares; insulin and 22:6,n-3 treated cells, dotted line, triangles.

Figure 4. Effect of 26S proteasome inhibitors on insulin and 22:6,n-3 regulation of SREBP-1. A. Primary rat hepatocytes were maintained overnight in Williams E medium containing 10 nM DEX and 20 mM lactate, but no serum, insulin or fatty acids. The next morning, cells were switched to medium supplemented with 25 mM glucose in the absence or presence of insulin (10 nM), 22:6,n-3 (100 μM), lactacystin (0.5 μM) or MG132 (10 μM). Lactacystin and MG132 are inhibitors of 26S proteasomal degradation of proteins. In the MG132 experiments cells were harvested after 12 of treatment. In the lactacystin studies, cells were harvested after 6 hrs of treatment. Microsomal and nuclear proteins were assayed for SREBP-1 and SREBP-2 by immunoblotting. **Immunoblots**: Representative immunoblots illustrate the effects of insulin, 22:6,n-3, lactacystin (upper blot) on SREBP-1 and SREBP-2 nuclear abundance or MG132 (lower blot) on SREBP-1 nuclear abundance. In the absence of insulin or 22:6,n-3, MG132 has no detectable effect on nSREBP-1c abundance. Results of the MG132 experiment are quantified and represented as Fold Change in nSREBP-1 induced by insulin or insulin + 22:6,n-3, mean ± SD, N=4. Results were evaluated by Student's T-test. *$p<0.001$ for Veh versus MG132 treated cells.

B. Time course of disappearance of SREBP-1 from hepatocyte nuclei. Primary rat hepatocytes were maintained overnight in Williams E medium containing 25 mM glucose, 10 nM DEX and 10 nM insulin to induce nSREBP-1. The next morning the media was changed to Williams E medium containing 10 nM DEX + 25 mM glucose with

insulin, without insulin or without insulin, but with 100 μM 22:6,n-3. Cells were harvested at 1.5, 6, 12 and 24 hrs for isolation of nuclear proteins. Levels of nSREBP-1 were quantified by immunoblotting. **C.** Primary rat hepatocytes were maintained overnight in Williams E medium containing 10 nM DEX, 25 mM glucose, 10 nM insulin to induce nSREBP-1. The next morning the media was changed to Williams E medium containing 10 nM DEX + 25 mM glucose with insulin, without insulin or without insulin, but with 100 μM 22:6,n-3. All three groups receive vehicle (Veh) or MG132 at 10 μM. Twelve hour later, cells were harvested for measurement of nSREBP-1 by immunoblotting. Results are expressed as Fold Change from control (Insulin treated with no MG132), mean ± SD, N=3. Results were evaluated by Student's T-test. *p<0.01, Veh vs MG132 treated cells. **D.** Cells were treated with insulin in the absence or presence of n-3 PUFA (100 μM) for 24 hours in the absence or presence of lactacystin. Nuclear and microsomal proteins were isolated and assayed for nSREBP-1, pSREBP-1 and nSREBP-2 by immunoblotting. The results are representative of 2 separate studies.

Figure 5. Role of Akt in regulating SREBP-1 nuclear abundance.

A. Time course of insulin-induction of AKT phosphorylation. Primary rat hepatocytes were maintained overnight in Williams E medium containing 10 nM DEX and 20 mM lactate, but with no serum, insulin or fatty acids. The next morning, cells were switched to medium supplemented 25 mM glucose and 10 nM insulin, or 25 mM glucose, 10 nM insulin and 100 μM 22:6,n-3. Cells were harvested at the times indicated for extraction of total hepatic proteins for measurement of total AKT and phospho-Akt (P-Akt) by immunoblotting. Results are presented as Fold Change in AKT Phosphorylation.

Results are normalized to the level of phospho-Akt in cells prior to insulin or 22:6,n-3 treatment. Mean ± SD, N=3. Results were evaluated by Student's T-test. *p<0.001; for insulin + 22:6,n-3 versus insulin treated cells. **Insert:** A representative immunoblot for P-AKT and total AKT at 1.5 hrs of treatment. **B.** Primary hepatocytes were either not infected (None) or infected with recombinant adenovirus expressing luciferase (Adv-Luc) [control], a kinase dead Akt, (Adv-Akt-KD) or a constitutively active AKT (Adv-Akt-CA). Cells were maintained in Williams E medium containing 10 nM DEX with no serum or insulin. Twenty four hours after infection, cells were harvested for analysis of microsomal and nuclear SREBP-1 by immunoblotting (**Insert**). Levels of microsomal (pSREBP-1) and nuclear (nSREBP-1) were quantified by immunoblotting. Results are expressed as Fold Change in nSREBP-1; normalized to levels of nSREBP-1 in non-infected cells, mean ± SD, N=3. Results were evaluated by Student's T-test. *p<0.01; for Ad-AKT-KD or AD-AKT-CA versus Ad-Luc infected cells. **C.** Primary hepatocytes were infected with recombinant adenovirus expressing luciferase (Ad-Luc) or constitutively active Akt (Adv-Akt-CA). Twenty four hours after infection, cells were treated with bovine serum albumin [BSA] (VEH)[white bar], insulin (10nM) [black bar] + BSA, 22:6,n-3 (100 μM) [gray bar] + BSA or a combination of insulin and 22:6,n-3 (Both)[pattern bar]. Six hours after treatment, cells were harvested for measurement of nuclear SREBP-1 abundance by immunoblotting. **D.** Results of 4 separate studies are quantified and normalized to the level of nSREBP-1 in vehicle treated AD-Luc infected cells. Results are expressed as Fold Change, mean ± SD, N=4. Results were evaluated by Student's T-test. *p<0.001; for 22:6,n-3 versus insulin treated cells.

Figure 6. Role of Erk in regulating SREBP-1 nuclear abundance.

A. Primary rat hepatocytes were maintained overnight in Williams E medium containing 20 mM lactate + 10 nM DEX with no serum or insulin. Cells were switched to medium supplemented with either 10 nM insulin (solid line) or insulin + 22:6,n-3 (dotted line). Cells were harvested at the times indicated for measurement of Erk phosphorylation by immunoblotting. **Insert**: Representative immunoblots illustrate the effects of insulin and 22:6,n-3 on total Erk (Erk) and Erk phosphorylation (P-Erk). Results are normalized to the level of Erk-phosphorylation in untreated primary hepatocytes; Fold Change in Erk Phosphorylation, mean ± SD, N=4. Results were evaluated by Student's T-test. *p<0.01; for insulin + 22:6,n-3 versus insulin treated cells.

B. Effect of palmitate, 20:5,n-3 and 22:6,n-3 on nSREBP-1 nuclear content (white bar) and Erk phosphorylation (black bar). Rat primary hepatocytes maintained overnight in Williams E medium containing 20 mM lactate + 10 nM DEX with no insulin or serum. Cells were switched to medium supplemented with 25 mM glucose, 10 nM insulin, 20 μM BSA in the absence or presence of fatty acids at 100 μM plus BSA (20 μM). Cells were harvested 24 hrs later for nuclear SREBP-1 (nSREBP-1) and Erk phosphorylation (P-Erk) by immunoblotting. Total Erk protein was unaffected by these treatments (not shown). Results are normalized to the level of nSREBP-1 or Erk-phosphorylation in untreated cells; mean ± SD, N=4. Results were evaluated by Student's T-test. *p<0.01; for Veh versus fatty acid treated cells.

Figure 7. The MEK inhibitors, PD98059 and U0126, rapidly abrogate 22:6,n-3 suppression of SREBP-1 nuclear abundance. A. Primary hepatocytes were

maintained overnight in Williams E medium containing 20 mM lactate, 10 nM insulin, 10 nM DEX, 20 μM BSA, without and with 100 μM 22:6,n-3. The next morning the medium was replaced with Williams E containing 25 mM glucose, 10 nM insulin, 10 nM DEX in the absence and presence of 22:6,n-3 (100 μM) or 10 μM PD98059 or U0126. After 30 minutes of treatment, cells were harvested for protein isolation and immunoblotting for nSREBP-1 and phospho-Erk (P-Erk) or Erk total protein (Erk). **B**. Immunoblots from the PD98059 study were quantified for the effects of 22:6,n-3 on Erk phosphorylation (P-Erk) and SREBP-1 nuclear abundance (nSREBP-1); 22:6,n-3 (white bar) or 22:6,n-3 + PD98059 (black bar). Results are normalized to the level of P-Erk and nuclear SREBP-1 (nSREBP-1) in cells receiving no 22:6,n-3 or PD98059; mean ± SD, N=4. Results are from 4 independent studies. Results were evaluated by Student's T-test. *$p<0.01$ for 22:6,n-3 versus 22:6,n-3 + PD98059 treated cells.

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Literature Cited.

1. Horton, J. D., J. L. Goldstein, and M. S. Brown. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* **109**: 1125-1131.
2. Sundqvist, A., Bengoechea-Alonso, M.T., Ye, X., Lukiyanchuk, Jin, J., Harper, J.W., and Ericsson, J. 2005. Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCGFBW7. *Cell Metabolism* **1**: 379-391.
3. Nagoshi, E., N. Imamoto, R. Sato, and Y. Yoneda. 1999. Nuclear import of sterol regulatory element-binding protein-2, a basic helix-loop-helix-leucine zipper (bHLH-Zip)-containing transcription factor, occurs through the direct interaction of importin beta with HLH-Zip. *Mol Biol Cell* **10**: 2221-2233.
4. Bennett, M. K., Toth, J.I., and Osborne, T.F. 2004. Selective association of sterol regulatory element binding protein isoforms with target promoters in vivo. *J Biol Chem* **279**: 37360-37367.
5. Hirano, Y., M. Yoshida, M. Shimizu, and R. Sato. 2001. Direct demonstration of rapid degradation of nuclear sterol regulatory element-binding proteins by the ubiquitin-proteasome pathway. *J Biol Chem* **276**: 36431-36437.
6. Adams, C. M., J. Reitz, J. K. De Brabander, J. D. Feramisco, L. Li, M. S. Brown, and J. L. Goldstein. 2004. Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. *J Biol Chem* **279**: 52772-52780.
7. Shimomura, I., Y. Bashmakov, S. Ikemoto, J. D. Horton, M. S. Brown, and J. L. Goldstein. 1999. Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* **96**: 13656-13661.
8. Schultz, J. R., H. Tu, A. Luk, J. J. Repa, J. C. Medina, L. Li, S. Schwendner, S. Wang, M. Thoolen, D. J. Mangelsdorf, K. D. Lustig, and B. Shan. 2000. Role of LXRs in control of lipogenesis. *Genes Dev* **14**: 2831-2838.
9. Repa, J. J., G. Liang, J. Ou, Y. Bashmakov, J. M. Lobaccaro, I. Shimomura, B. Shan, M. S. Brown, J. L. Goldstein, and D. J. Mangelsdorf. 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* **14**: 2819-2830.
10. Fleischmann, M., and P. B. Iynedjian. 2000. Regulation of sterol regulatory-element binding protein 1 gene expression in liver: role of insulin and protein kinase B/cAkt. *Biochem J* **349**: 13-17.
11. Ono, H., Shimano, H, Katagiri, H, Yahagi, N, Sakoda, H, Onishi, Y, Anai, M, Ogihara, T, Fujishiro, M, Viana, AYI, Fukushima, Y, Abe, M, Shojima, N, Kikuchi, M, Yamada, N, Oka, Y and Asano, T. 2003. Hepatic AKT activation induces marked hypoglycemia, hepatomegaly and hypertriglyceridemia with sterol regulatory element binding protein involvement. *Diabetes* **52**: 2905-2913.

12. Ribaux, P. G., and P. B. Iynedjian. 2003. Analysis of the role of protein kinase B (cAKT) in insulin-dependent induction of glucokinase and sterol regulatory element-binding protein 1 (SREBP1) mRNAs in hepatocytes. *Biochem J* **376**: 697-705.
13. Yabe, D., R. Komuro, G. Liang, J. L. Goldstein, and M. S. Brown. 2003. Liver-specific mRNA for Insig-2 down-regulated by insulin: implications for fatty acid synthesis. *Proc Natl Acad Sci U S A* **100**: 3155-3160.
14. Keeton, A. B., K. D. Bortoff, J. L. Franklin, and J. L. Messina. 2005. Blockade of rapid versus prolonged ERK1/2 activation has differential effects on insulin-induced gene expression. *Endocrinology* **146**: 2716-2725.
15. Jump, D. B. 2002. Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr Opin Lipidol* **13**: 155-164.
16. Jump, D. B. 2004. Fatty acid regulation of gene transcription. *Cric. Rev. Clin. Lab Sci.* **41**: 41-78.
17. Xu, J., M. T. Nakamura, H. P. Cho, and S. D. Clarke. 1999. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *J Biol Chem* **274**: 23577-23583.
18. Hannah, V. C., J. Ou, A. Luong, J. L. Goldstein, and M. S. Brown. 2001. Unsaturated fatty acids down-regulate srebp isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J Biol Chem* **276**: 4365-4372.
19. Mater, M. K., A. P. Thelen, D. A. Pan, and D. B. Jump. 1999. Sterol response element-binding protein 1c (SREBP1c) is involved in the polyunsaturated fatty acid suppression of hepatic S14 gene transcription. *J Biol Chem* **274**: 32725-32732.
20. Xu, J., M. Teran-Garcia, J. H. Park, M. T. Nakamura, and S. D. Clarke. 2001. Polyunsaturated fatty acids suppress hepatic sterol regulatory element-binding protein-1 expression by accelerating transcript decay. *J Biol Chem* **276**: 9800-9807.
21. Deng, X., L. M. Cagen, H. G. Wilcox, E. A. Park, R. Raghow, and M. B. Elam. 2002. Regulation of the rat SREBP-1c promoter in primary rat hepatocytes. *Biochem Biophys Res Commun* **290**: 256-262.
22. Cagen, L. M., X. Deng, H. G. Wilcox, E. A. Park, R. Raghow, and M. B. Elam. 2005. Insulin activates the rat sterol-regulatory-element-binding protein 1c (SREBP-1c) promoter through the combinatorial actions of SREBP, LXR, Sp-1 and NF-Y cis-acting elements. *Biochem J* **385**: 207-216.
23. Worgall, T. S., S. L. Sturley, T. Seo, T. F. Osborne, and R. J. Deckelbaum. 1998. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem* **273**: 25537-25540.
24. Chen, G., G. Liang, J. Ou, J. L. Goldstein, and M. S. Brown. 2004. Central role for liver X receptor in insulin-mediated activation of Srebp-1c

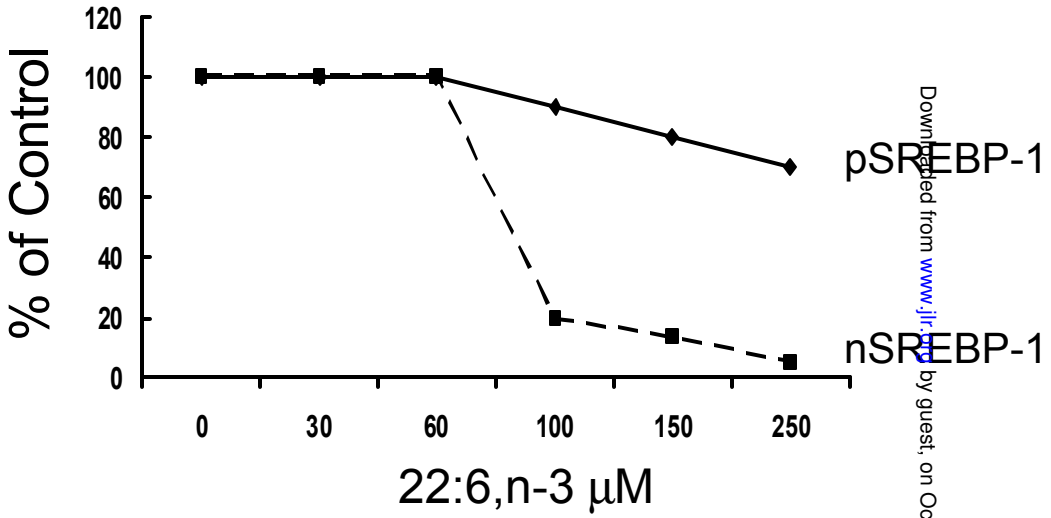
transcription and stimulation of fatty acid synthesis in liver. *Proc Natl Acad Sci U S A* **101**: 11245-11250.

25. Nakatani, T., Katsumata, A., Miura, S., Kamei, Y., and Ezaki, O. 2005. Effects of fish oil feeding and fasting on LXRalpha/RXRalpha binding to LXRE in the SREBP-1c promoter of mouse liver. *Biochimica et Biophysica Acta* **1736**: 77-86.
26. Ou, J., H. Tu, B. Shan, A. Luk, R. A. DeBose-Boyd, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 2001. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc Natl Acad Sci U S A* **98**: 6027-6032.
27. Pawar, A., J. Xu, E. Jerks, D. J. Mangelsdorf, and D. B. Jump. 2002. Fatty acid regulation of liver X receptors (LXR) and peroxisome proliferator-activated receptor alpha (PPARalpha) in HEK293 cells. *J Biol Chem* **277**: 39243-39250.
28. Pawar, A., Botolin, D., Mangelsdorf, D.J., and Jump, D.B. 2003. The role of liver X receptor-alpha (LXR-alpha) in the fatty acid regulation of hepatic gene expression. *J Biol Chem* **278**: 40736-40743.
29. Pan, D. A., M. K. Mater, A. P. Thelen, J. M. Peters, F. J. Gonzalez, and D. B. Jump. 2000. Evidence against the peroxisome proliferator-activated receptor alpha (PPARalpha) as the mediator for polyunsaturated fatty acid suppression of hepatic L-pyruvate kinase gene transcription. *J Lipid Res* **41**: 742-751.
30. Wang, Y., Botolin, D., Christian, B., Busik, C., Xu, J. & Jump, D.B. 2005. Tissue-specific, nutritional and developmental regulation of rat fatty acid elongases. *J Lipid Res* **46**: 706-715.
31. Botolin, D., and D. B. Jump. 2003. Selective proteolytic processing of rat hepatic sterol regulatory element binding protein-1 (SREBP-1) and SREBP-2 during postnatal development. *J Biol Chem* **278**: 6959-6962.
32. Wrede, C. E., L. M. Dickson, M. K. Lingohr, I. Briaud, and C. J. Rhodes. 2002. Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *J Biol Chem* **277**: 49676-49684.
33. Sprecher, H. 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochimica et Biophysica Acta* **1486**: 219-231.
34. Keeton, A. B., K. D. Bortoff, W. L. Bennett, J. L. Franklin, D. Y. Venable, and J. L. Messina. 2003. Insulin-regulated expression of Egr-1 and Krox20: dependence on ERK1/2 and interaction with p38 and PI3-kinase pathways. *Endocrinology* **144**: 5402-5410.
35. Pegorier, J. P., C. Le May, and J. Girard. 2004. Control of gene expression by fatty acids. *J Nutr* **134**: 2444S-2449S.
36. Ren, B., A. P. Thelen, J. M. Peters, F. J. Gonzalez, and D. B. Jump. 1997. Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor alpha. *J Biol Chem* **272**: 26827-26832.

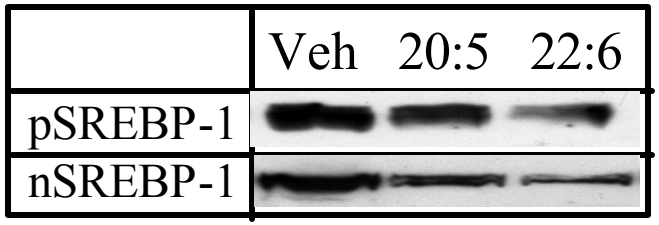
37. Sundqvist, A., and J. Ericsson. 2003. Transcription-dependent degradation controls the stability of the SREBP family of transcription factors. *Proc Natl Acad Sci U S A* **100**: 13833-13838.
38. Kim, K. H., Song, M.J., Yoo, E.J., Choe, S.S., Park, S.D. and Kim, J.B. 2004. Regulatory role of GSK3 for transcriptional activity of ADD1/SREBP1c. *J Biol Chem* **279**: 51999-52006.
39. Roth, G., J. Kotzka, L. Kremer, S. Lehr, C. Lohaus, H. E. Meyer, W. Krone, and D. Muller-Wieland. 2000. MAP kinases Erk1/2 phosphorylate sterol regulatory element-binding protein (SREBP)-1a at serine 117 in vitro. *J Biol Chem* **275**: 33302-33307.

Figure 1 A-C

A.



B.



C.

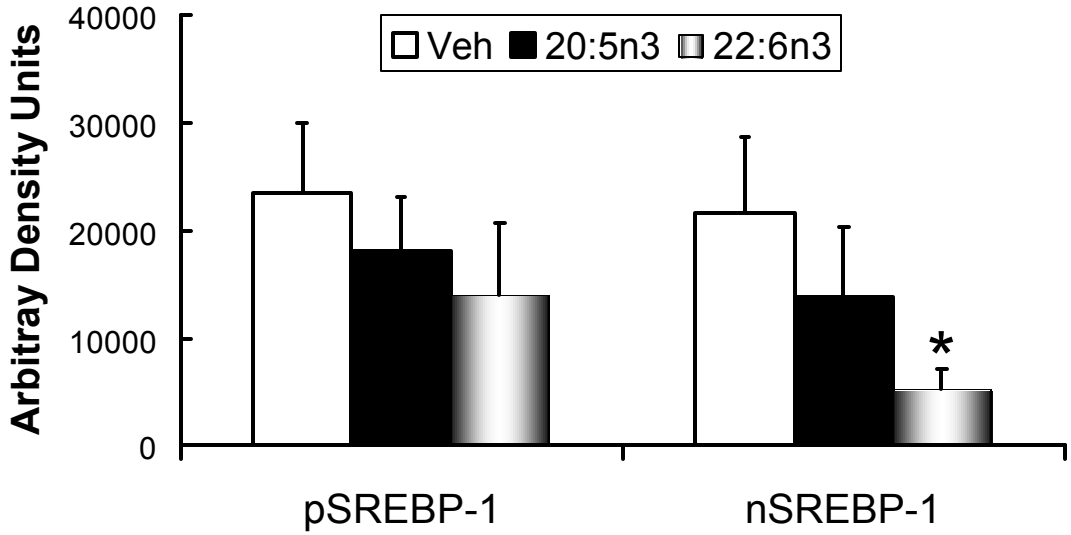


Figure 2A

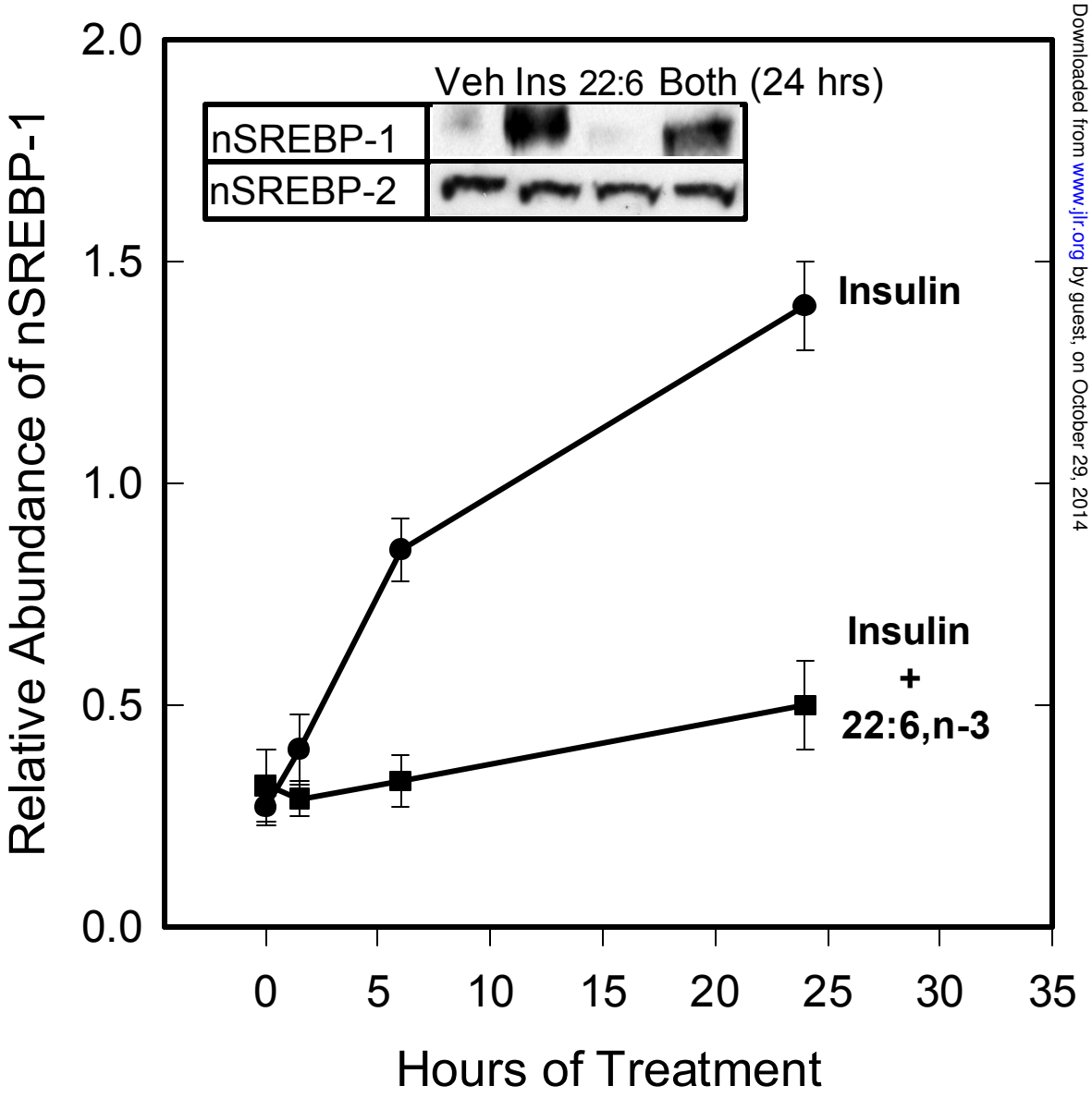


Figure 2B

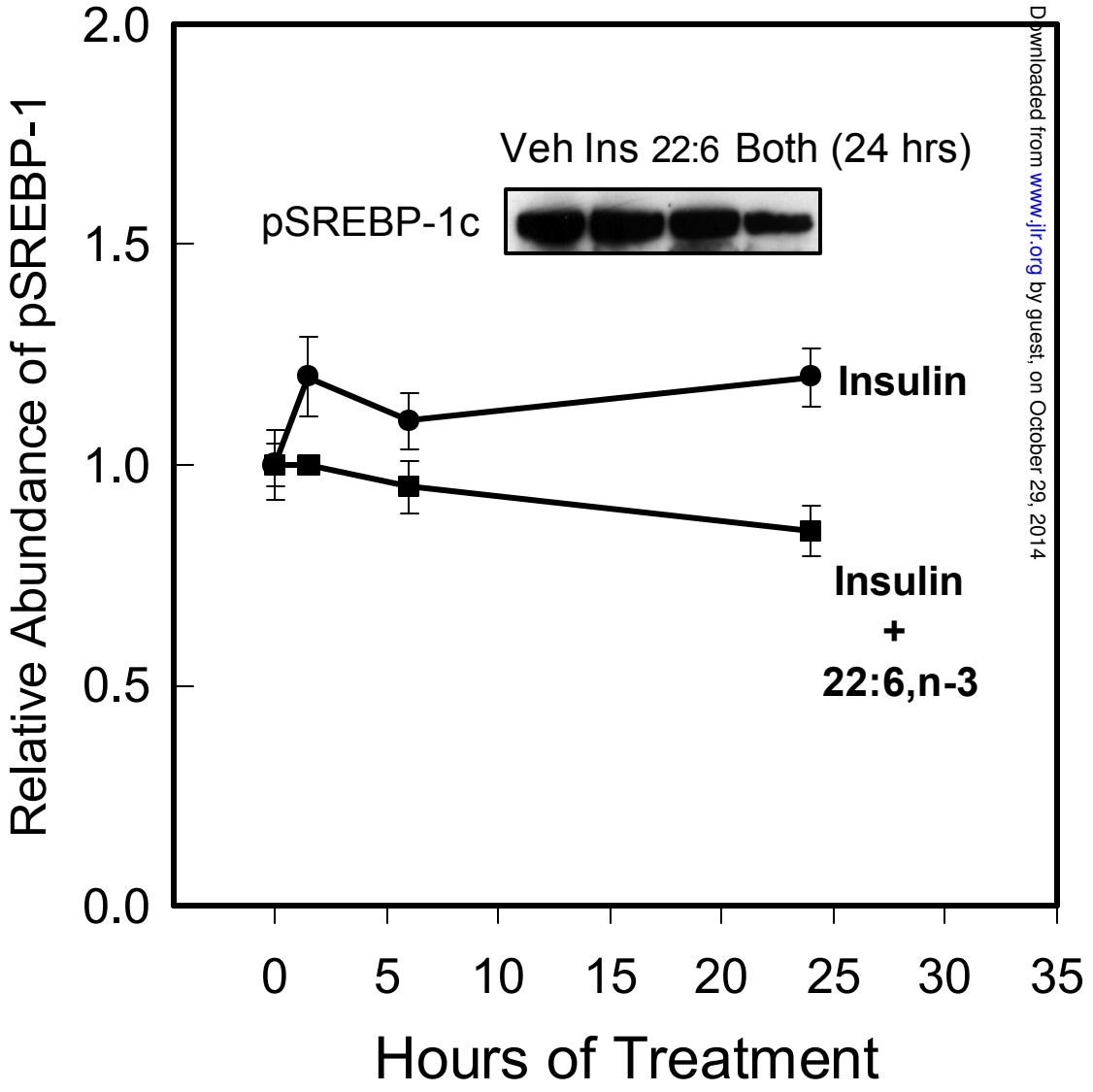


Figure 2C

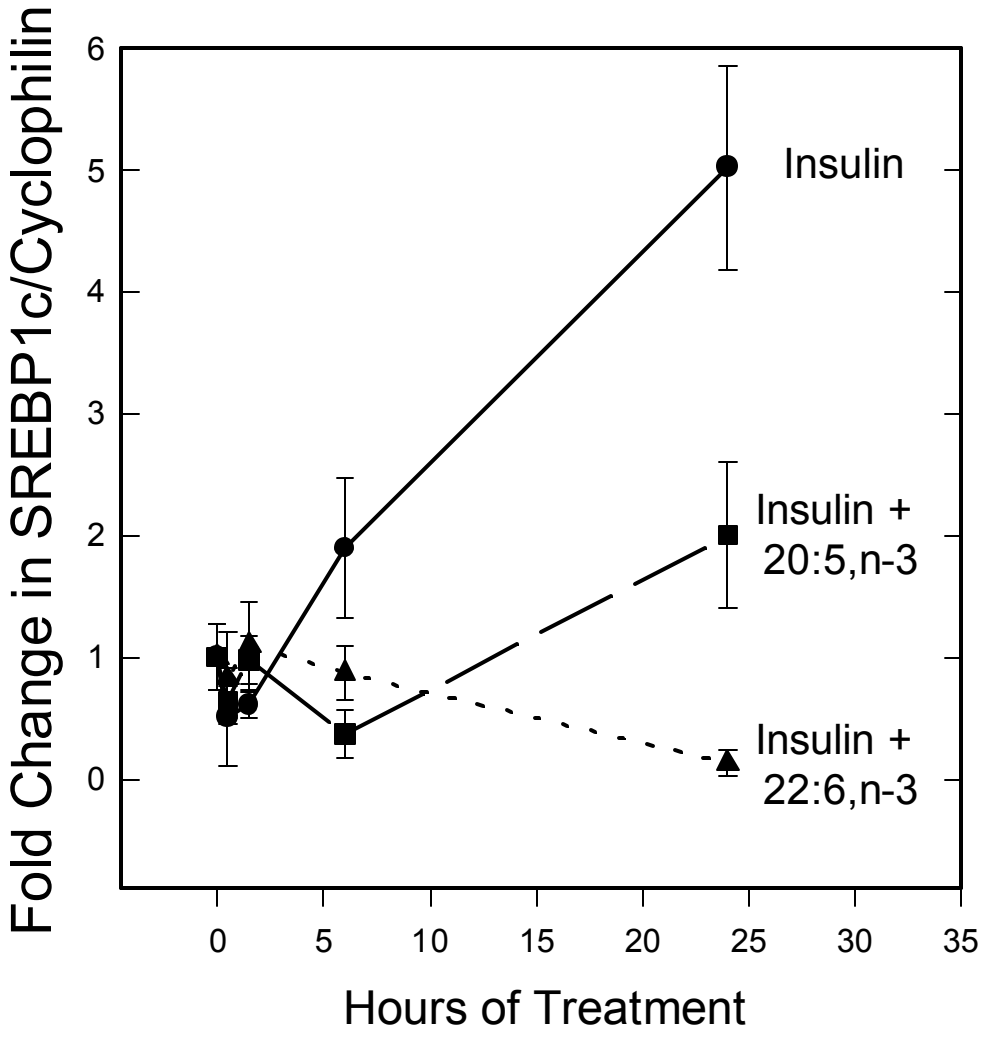
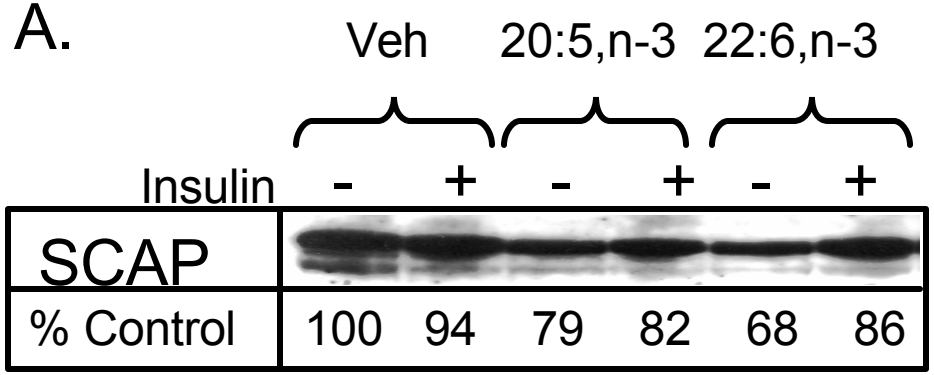


Figure 3

A.



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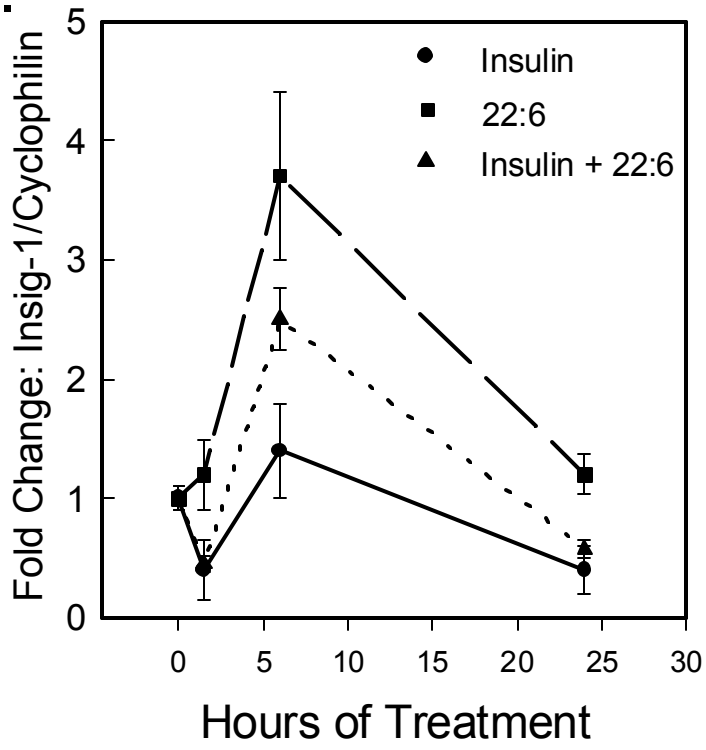

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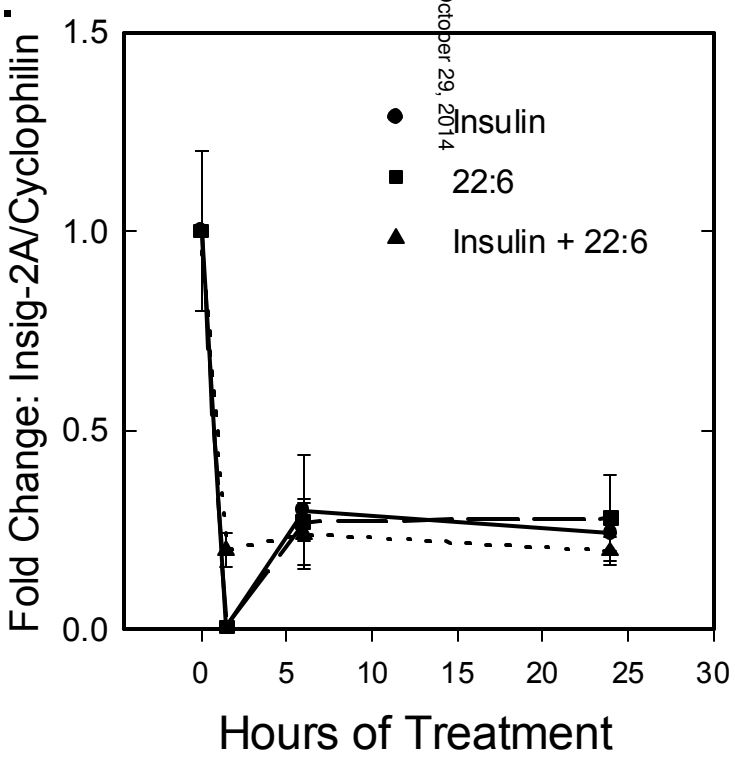
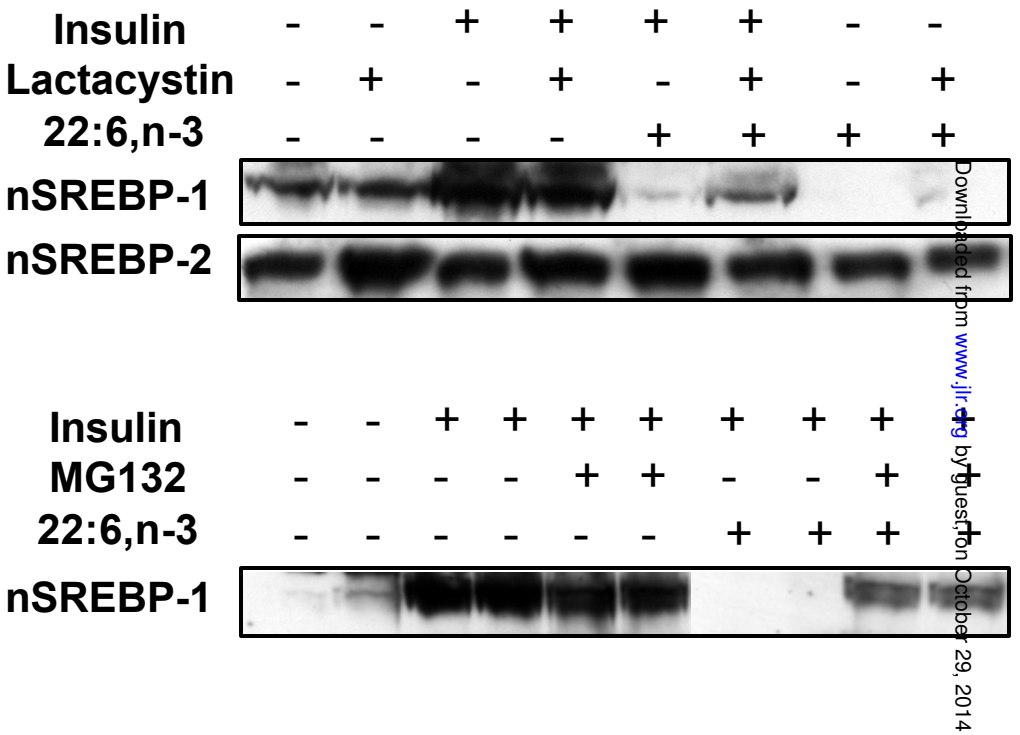


Figure 4A



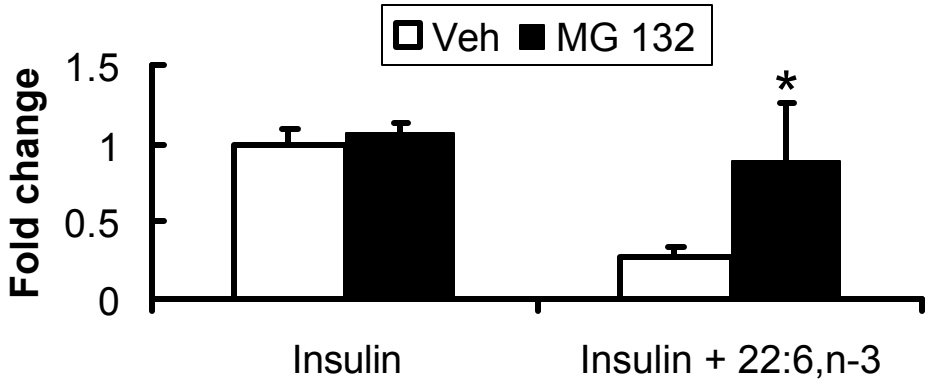


Figure 4B and C.

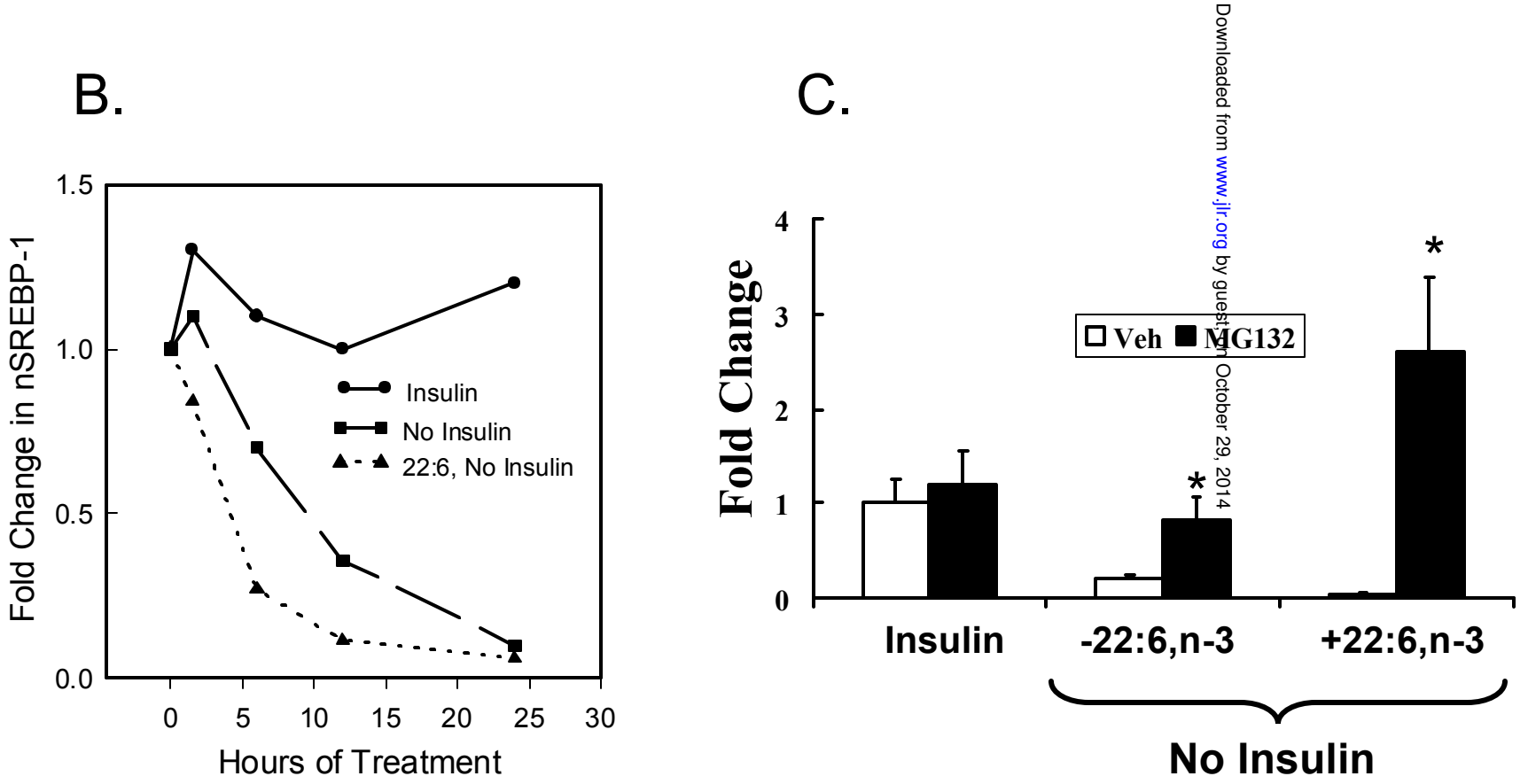
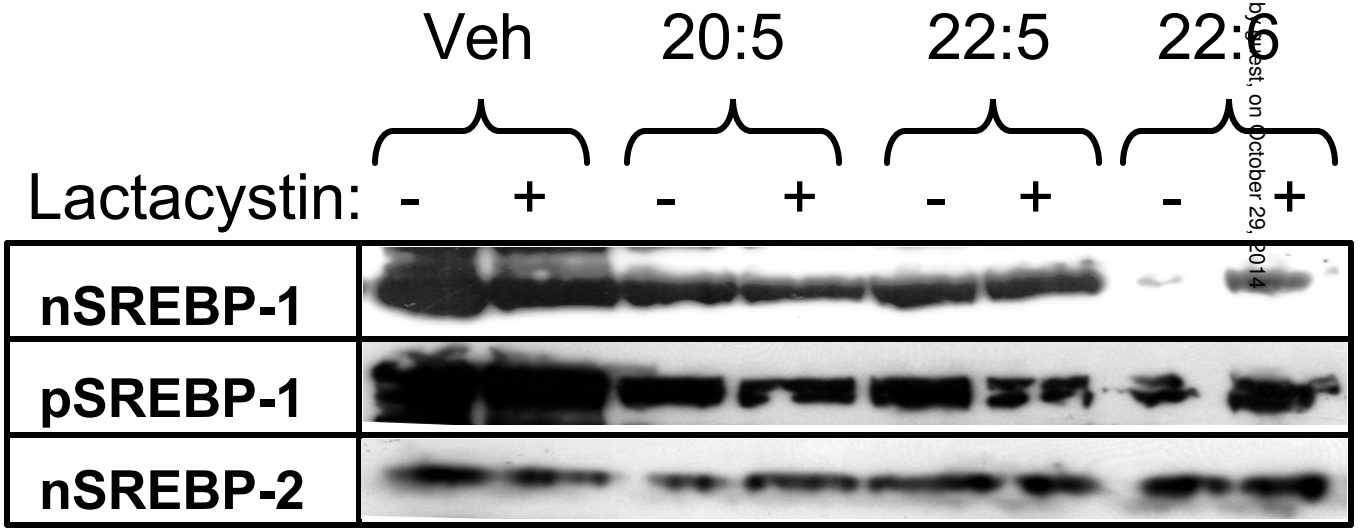
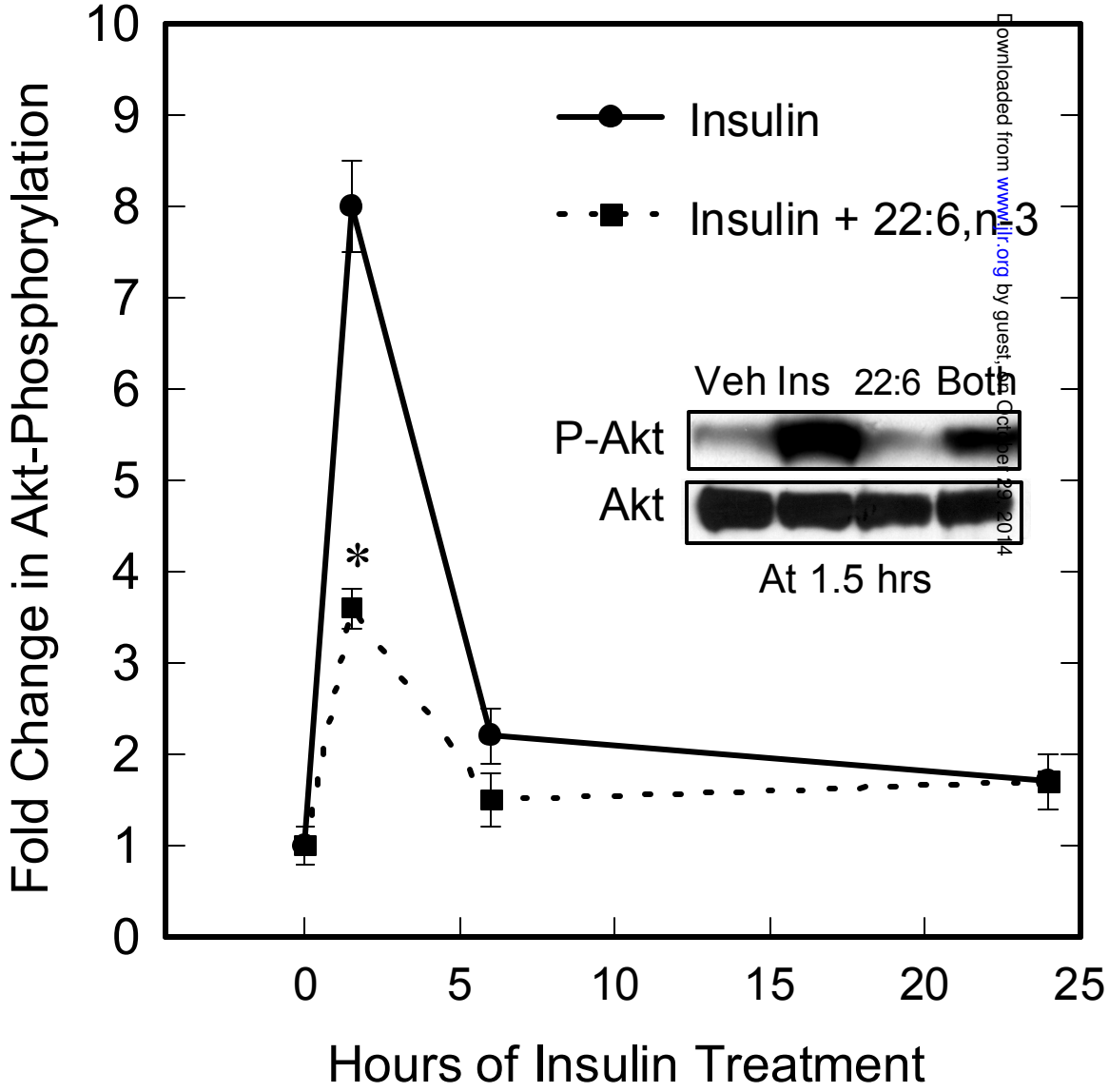


Figure 4D



A.

Figure 5A



B.

Figure 5B

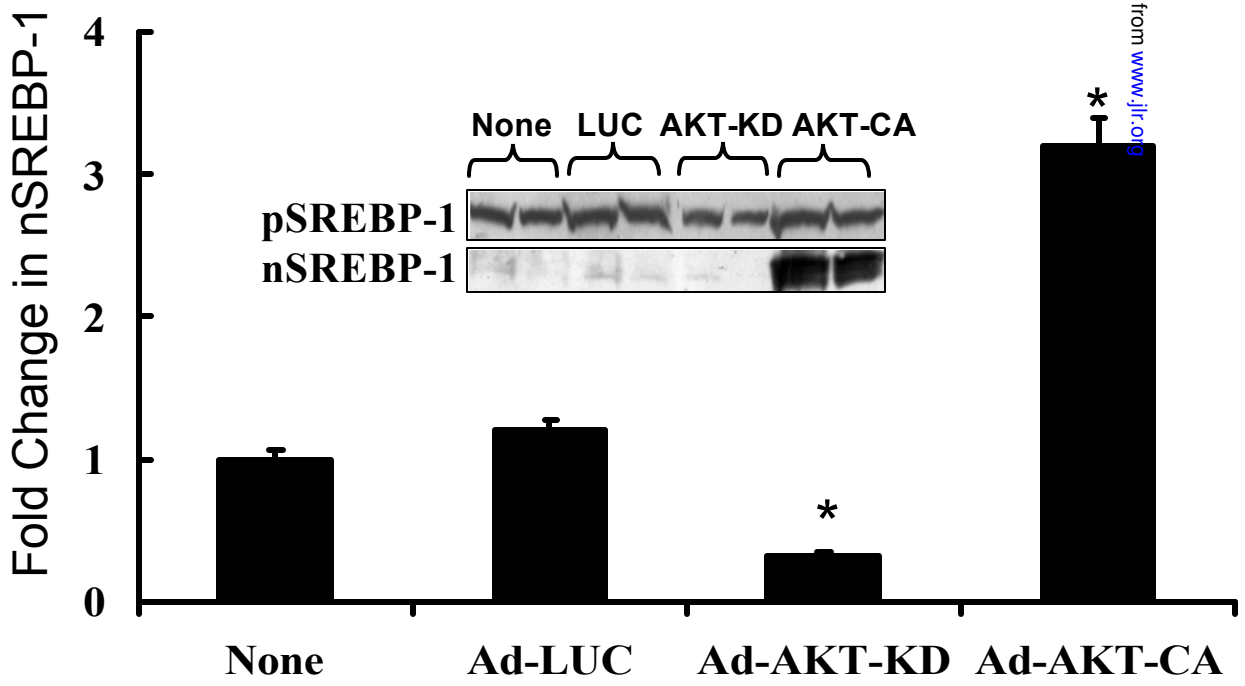


Figure 5 C & D

C.

D.

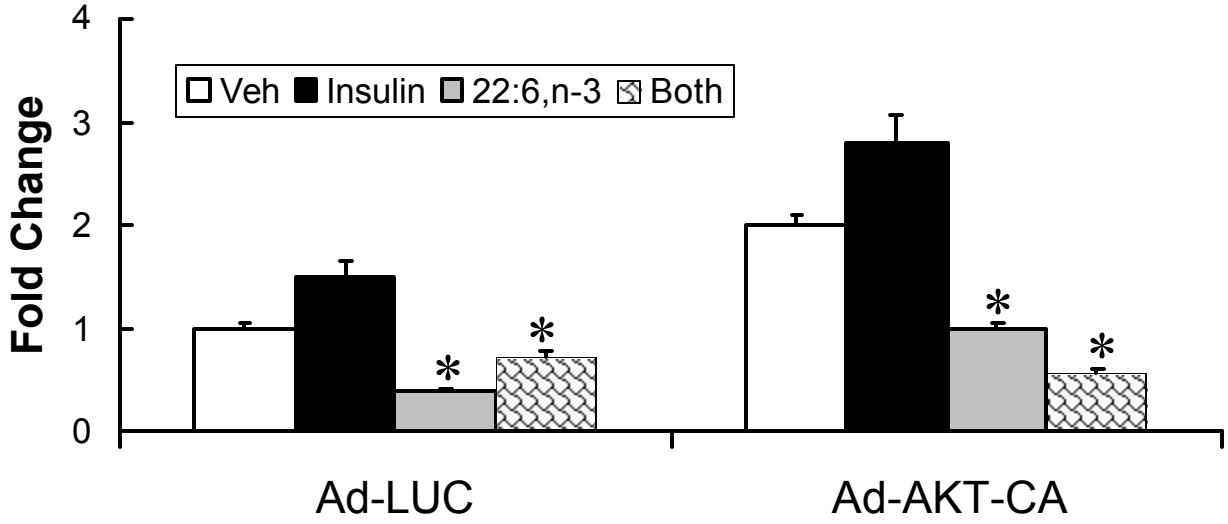


Figure 6A

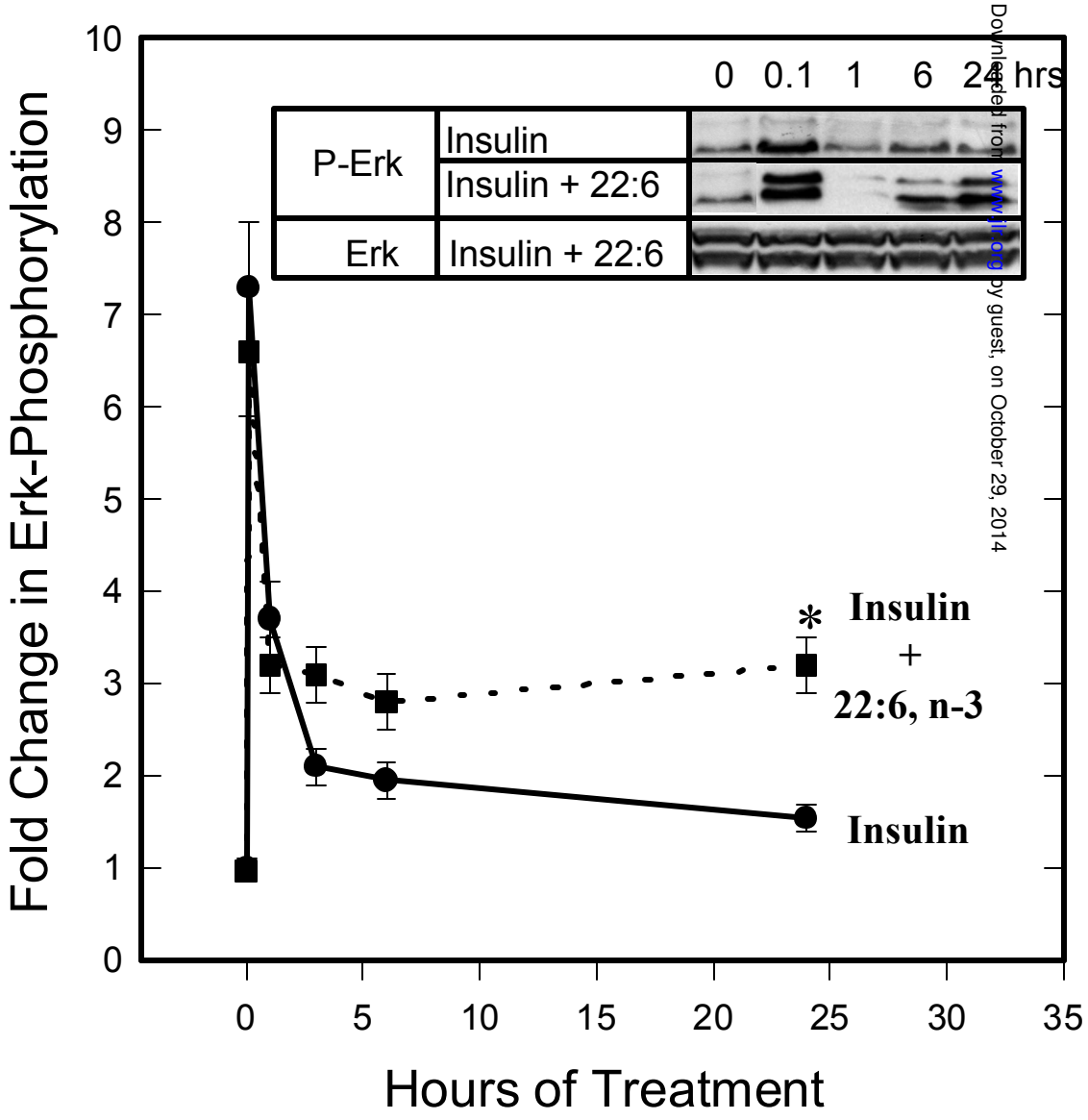


Figure 6B

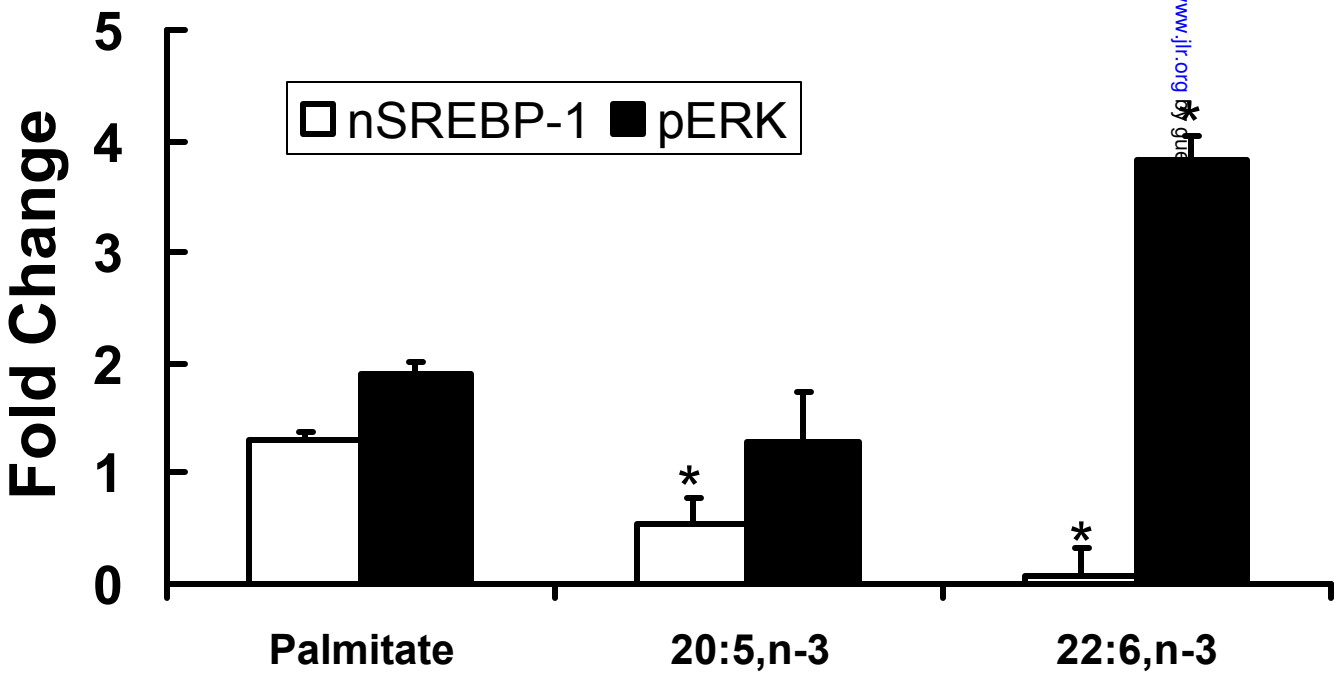
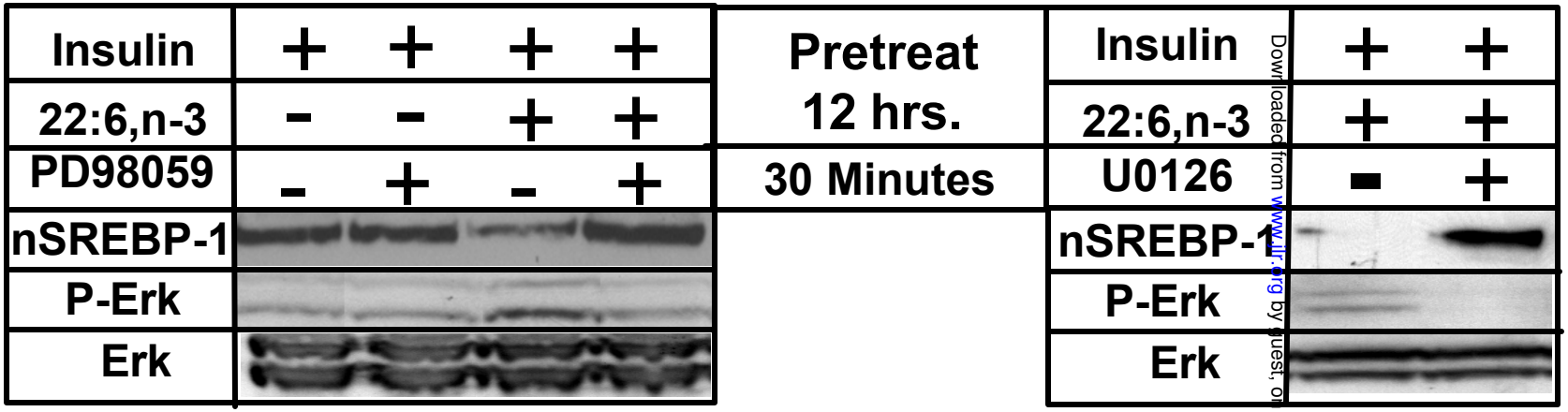


Figure 7

A.



B.