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THE INSULIN RECEPTOR TRANSLOCATES TO THE NUCLEUS TO REGULATE CELL PROLIFERATION IN LIVER

Maria J. Amaya¹, André G. Oliveira², Erika S. Guimarães², Marisa C. F. Casteluber², Sandhra M. Carvalho³, Lidia M. Andrade^{2,4}, Mauro C. X. Pinto², Albert Mennone¹, Cleida A. Oliveira⁵, Rodrigo R. Resende⁶, Gustavo B. Menezes⁵, Michael H. Nathanson¹, and M. Fatima Leite^{2,7}

¹Section of Digestive Diseases, Department of Internal Medicine, Yale University. 333 Cedar Street. PO Box 208019. New Haven-CT, 06520-8019, USA

²Department of Physiology and Biophysics, Federal University of Minas Gerais. Av. Antônio Carlos, 6627. Belo Horizonte-Minas Gerais. CEP: 31270-901, Brazil

³School of Engineering, Federal University of Minas Gerais, Av. Antônio Carlos, 6627. Belo Horizonte-Minas Gerais. CEP: 31270-901, Brazil

⁴René Rachou Research Center, Oswaldo Cruz Foundation. Av. Augusto de Lima, 1715. Belo Horizonte-Minas Gerais. CEP: 30190-002, Brazil

⁵Department of Morphology, Federal University of Minas Gerais. Av. Antônio Carlos, 6627. Belo Horizonte-Minas Gerais. CEP: 31270-901, Brazil

⁶Department of Biochemistry and Immunology, Federal University of Minas Gerais. Av. Antônio Carlos, 6627. Belo Horizonte-Minas Gerais. CEP: 31270-901, Brazil.

⁷Howard Hughes Medical Institute

Abstract

Insulin's metabolic effects in the liver are widely appreciated, but insulin's ability to act as a hepatic mitogen is less well understood. Because the Insulin Receptor (IR) can traffic to the nucleus, and calcium (Ca^{2+}) signals within the nucleus regulate cell proliferation, we investigated whether insulin's mitogenic effects result from activation of Ca^{2+} signaling pathways by IRs within the nucleus. Insulin-induced increases in Ca^{2+} and cell proliferation depended upon clathrin- and caveolin-dependent translocation of the IR to the nucleus, as well as upon formation of inositol 1,4,5,-trisphosphate (InsP_3) in the nucleus, whereas insulin's metabolic effects did not depend on either of these events. Moreover, liver regeneration after partial hepatectomy also depended upon formation of InsP_3 in the nucleus but not the cytosol, whereas hepatic glucose metabolism was not affected by buffering InsP_3 in the nucleus. *Conclusion:* These findings provide evidence that insulin's mitogenic effects are mediated by a subpopulation of IRs that traffic to the nucleus to locally activate InsP_3 -dependent Ca^{2+} signaling pathways. The steps along this signaling pathway reveal a number of potential targets for therapeutic modulation of liver growth in health and disease.

Keywords

Receptor Tyrosine Kinase; calcium signaling; inositol 1,4,5,-trisphosphate; liver regeneration

Liver regeneration is a carefully orchestrated process regulated by cytokines, growth factors, hormones, and neurotransmitters, which act in concert to restore liver mass and function within days after tissue loss (1, 2). This process relies not only on proliferative cascades, in which hepatocytes switch from a quiescent to a proliferative phenotype (1, 2), but also on metabolic pathways that help maintain cellular homeostasis after liver injury (3). Growth factors are particularly important for this process, and insulin specifically regulates both metabolism and proliferation in the liver (4, 5). However, insulin's effects on liver regeneration are less well understood than those of other growth factors such as Epidermal Growth Factor (EGF) and Hepatocyte Growth Factor (HGF) (6, 7). Insulin acts through the Insulin Receptor (IR), a heterotetrameric receptor tyrosine kinase (RTK) composed of two extracellular α subunits, which have ligand-binding activity, and two transmembrane β subunits that possess tyrosine kinase activity (8). Once insulin binds to the IR, protein tyrosine kinase is activated, resulting in phosphorylation of the tyrosine residues within the β subunit. This in turn leads to the recruitment of several adaptor proteins, including *src*-homology 2 domain (SH2)-containing proteins such as phosphatidylinositol 3-kinase (PI3K) and Phospholipase C (PLC) (9). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂), resulting in the formation of diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol-1,4,5-trisphosphate (InsP₃), which promotes Ca²⁺ release from intracellular stores upon binding to the InsP₃ receptor (InsP₃R) (10).

Several RTKs, including the IR and the receptors for EGF, HGF, and Fibroblast Growth Factor (FGF) have been found in the nucleus (11-15). Evidence suggests that the IR, like the HGF receptor *c-met* (14), translocates to the nucleus upon ligand stimulation in order to selectively hydrolyze nuclear PIP₂ and locally generate InsP₃-dependent Ca²⁺ signals there (11). Additionally, nucleoplasmic rather than cytosolic Ca²⁺ is important for cellular proliferation, and is necessary in particular for progression through early prophase (16). However, metabolic effects of insulin result from cytosolic rather than intranuclear events, typified by activation of Akt (17). Therefore, we examined whether the cytosolic and nuclear effects of IR are mediated separately, and whether the subpopulation of IRs reaching the nucleus upon insulin stimulation locally induces InsP₃-dependent Ca²⁺ signals in order to regulate the proliferative effects of insulin.

MATERIALS AND METHODS

Cells and cell culture

The liver cancer cell line SkHep-1 was obtained from the American Type Culture Collection (ATCC). Cells were cultured at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 units/mL penicillin, and 50 g/mL streptomycin (Invitrogen, Grand Island, NY).

Animals

Male Holtzman rats (70 g) obtained from CEBIO (Federal University of Minas Gerais, Brazil) or from Harlan Laboratories (South Easton, MA) were used for all studies. Animals were maintained on a standard diet and housed under a 12 h light-dark cycle. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (publication 86-23, revised 1985).

Immunofluorescence

SkHep-1 cells plated onto coverslips were fixed with 4% paraformaldehyde. Confocal immunofluorescence was performed as previously described (18, 19).

Immunoblots

SkHep-1 cell and Holtzman rat hepatocyte immunoblots and separation of nuclear and non nuclear protein extracts were carried out as previously described (11).

Cell surface biotinylation

Cell surface biotinylation and streptavidin pull-down were performed, with modifications, as previously described (14).

InsP₃ buffer constructs

Plasmids were generated (14) and adenoviral constructs were amplified and purified as previously described (20).

Detection of Ca²⁺ signals

Ca²⁺ signals were detected and measured by time lapse confocal microscopy as described (14, 18, 19).

Transfection of siRNA

Validated siRNAs for clathrin heavy chain and caveolin-1 were obtained from Ambion. SkHep-1 cells were transfected with 5nM of each siRNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Grand Island, NY). Cells were used 48 h after transfection.

Measurement of BrdU incorporation

Cell proliferation was measured by BrdU incorporation using an enzyme linked immunosorbent assay (Roche Applied Science, Indianapolis, IN), according to the manufacturer's instructions.

Partial Hepatectomy and *In vivo* proliferation assay

Two-thirds hepatectomy (PH) was performed on adult male Holtzman rats as described (21).

Immunohistochemistry

Immunohistochemistry was performed following standard methods for microwave antigen retrieval (22).

Quantification of plasmatic glucose

Glucose content in the blood was measured using an enzymatic colorimetric assay method (Analisa, Belo Horizonte, Brazil), according to the manufacturer's instructions.

Quantification of Glycogen in Liver

The glycogen content from liver samples was determined by a phenol-sulfuric acid method as described by Dubois *et al.* (23) with modifications.

Statistical Analysis

Results are expressed as mean values \pm S.D. Prism (GraphPad, La Jolla, CA) was used for data analysis. Groups of data were compared using Student's t test or one-way ANOVA (which was used because data sets included only one independent variable) followed by Bonferroni post-tests, and p value <0.05 was taken to indicate statistical significance.

Detailed and additional methods are available in Supporting Materials and Methods

RESULTS

The Insulin Receptor (IR) translocates to the nucleus to initiate nuclear InsP_3 -dependent Ca^{2+} signals

Translocation of the IR to the nucleus has been observed in primary rat hepatocytes (11). To investigate whether the IR translocates to the nucleus in the SkHep-1 human hepatoma cell line as well, cells were analyzed by confocal immunofluorescence microscopy, to monitor the localization of the IR before and after insulin stimulation. This liver cell line was used because, as in primary hepatocytes, it contains Ca^{2+} signaling machinery in both the cytoplasm and the nucleus (18). Moreover, SkHep-1 cells functionally express particularly well studied G-protein-coupled receptors in hepatocytes such as the V1a vasopressin receptor (14, 24) and the purinergic P2Y receptor (25), as well as receptors for HGF (14), EGF (12), and the IR (11), which are important RTKs in the process of liver regeneration (2). Furthermore, the gamma-1 isoform of PLC, which is activated by growth factors, is also present in these cells (14). In addition, SkHep-1 cells express the type II InsP_3R (18), which is the most abundant isoform of this Ca^{2+} release channel in hepatocytes (26). Furthermore, the use of this cell line would facilitate transient transfection studies (11). Prior to stimulation with insulin, the IR was preferentially localized to the plasma membrane (PM) and nearly absent from the nucleus. After 5 min of insulin (10 nM) exposure, the IR was diffusely distributed in the cytoplasm and in the nuclear interior, and nuclear labeling was further intensified after 10 min of stimulation (Fig. 1A and B). To confirm the immunofluorescence results, immunoblots of non-nuclear and nuclear fractions of SkHep-1 cells were performed before and 10 min after insulin stimulation. The purity of the nuclear fractions was confirmed by the presence of the nuclear markers Lamin B1 and Histone H3 and the absence of the cytosolic markers Na^+/K^+ ATPase, GAPDH and α -tubulin (Fig. 1C). Additionally, Immuno-electron Microscopy for GAPDH and α -tubulin revealed the expression of these markers exclusively in the cytosol of intact SkHep-1 cells and their absence from intact isolated nuclei (Supporting Fig.1) Similar results were found in samples from isolated rat hepatocytes (not shown). IR was detected in non-nuclear fractions and at low levels in the nuclear fractions before stimulation. However, there was an increase in IR expression in the nuclear fractions after 10 min of insulin treatment (Fig. 1C). In order to determine whether the receptors that reach the nucleus originate at the plasma membrane, cell surface biotinylation and subsequent streptavidin pull-down of non-nuclear and nuclear SkHep-1 fractions were performed. Biotinylated IR was found in the nuclear fractions only after stimulation with insulin for 10 min (Fig. 1D). Together, these results show that the IR translocates from the plasma membrane to the nucleus of SkHep-1 cells upon insulin stimulation, similar to what is observed in primary rat hepatocytes (11).

To verify the relative contribution of nuclear versus cytosolic InsP_3 to insulin-induced Ca^{2+} signals, we used specific adenoviral mRFP-tagged nuclear or cytosolic- InsP_3 buffers, which contain the ligand binding domain (residues 224-605) of the human Type 1 InsP_3R with either a nuclear localization sequence (InsP_3 -Buffer-NLS), or a nuclear exclusion signal (InsP_3 -buffer-NES), respectively (14, 20). SkHep-1 cells loaded with the Ca^{2+} -sensitive dye Fluo-4/AM were examined by time-lapse confocal microscopy under control, nuclear and cytosolic InsP_3 buffering conditions. Nuclear and cytosolic Ca^{2+} signals were monitored during insulin (10 nM) stimulation. InsP_3 -Buffer-NLS and InsP_3 -Buffer-NES were correctly localized in the nucleus and in the cytosol, respectively (Fig. 2A). In control cells, insulin-induced Ca^{2+} signals occurred in the nucleus and in the cytosol. However, the Ca^{2+} increase occurred first in the nucleus (Fig. 2A and B). Both nuclear and cytosolic Ca^{2+} signals were nearly eliminated by buffering InsP_3 in the nucleus (Fig. 2A, C and E); nuclear Ca^{2+} signals were not affected in the presence of the cytosolic InsP_3 buffer, while cytosolic Ca^{2+} signals had a minimal decrease (Fig. 2A, D and E). These results are similar to previous findings in

SkHep-1 cells (11). Collectively, these observations demonstrate that insulin promotes IR translocation to the nucleus and initiation of Ca^{2+} signals dependent on nuclear InsP_3 .

Insulin-induced cell proliferation depends upon nuclear InsP_3

Insulin regulates viability, growth and proliferation of primary hepatocytes and hepatoma cell lines (4, 27), and nuclear rather than cytosolic Ca^{2+} is required for cell proliferation (16). To verify whether nuclear InsP_3 is the upstream regulator of insulin-induced cell proliferation, SkHep-1 cells were synchronized in G0 by serum withdrawal, transfected with InsP_3 -Buffer-NLS, and assayed for bromodeoxyuridine (BrdU) incorporation. Insulin, 10% fetal bovine serum and HGF each induced significant increases in BrdU uptake when compared to unstimulated control cells, as expected. However, BrdU uptake was reduced in cells expressing InsP_3 -Buffer-NLS, relative to control cells treated with insulin. Nuclear InsP_3 -buffered cells treated with insulin also had significantly smaller BrdU uptake than control cells stimulated with insulin. BrdU uptake in InsP_3 -Buffer-NLS cells stimulated with insulin was not significantly higher than in untreated InsP_3 -Buffer-NLS cells (Fig. 2F). Together, these results indicate that formation of InsP_3 in the nucleus is required for insulin-induced cell proliferation.

Insulin-induced proliferation depends on IR translocation to the nucleus

Upon insulin stimulation, the IR undergoes endocytosis through the classic clathrin-dependent pathway, like other RTKs (28). However, a subpopulation of IRs on the PM is associated with caveolin-enriched membrane domains (29). In order to determine whether clathrin heavy chain and/or caveolin-1 are necessary to mediate IR translocation from the plasma membrane to the nucleus, we used specific siRNAs that allowed a knockdown of 97% in both clathrin heavy chain (cla) and caveolin-1 (cav) expression, compared to scrambled siRNA transfected cells (Fig. 3A-D). Immunoblots of non-nuclear and nuclear fractions showed that silencing of cav caused a decrease in nuclear IR by 46.5% when compared to scrambled siRNA transfected cells stimulated with 10 nM insulin. Silencing of cla caused a 24.7% decrease in nuclear IR as compared to scrambled siRNA transfected cells stimulated with insulin (10 nM), which was marginally significant ($p=0.08$). Furthermore, simultaneous silencing of both proteins had an additive effect, causing a decrease in nuclear IR by 65.8% when compared to scrambled siRNA transfected cells stimulated with insulin (Fig. 3E and F). These observations suggest that these two proteins act in concert to mediate the translocation of the IR to the nucleus upon insulin stimulation. To determine whether translocation of IR to the nucleus is necessary for insulin-induced cell proliferation, cells were assayed for BrdU uptake as described above in the presence of each or both siRNAs. The presence of either cla or cav siRNA decreased BrdU uptake, compared to scrambled siRNA transfected cells treated with insulin (Fig. 3G). Cla or cav siRNA transfected cells treated with insulin also had reduced BrdU uptake compared to scrambled siRNA transfected cells treated with insulin. Similarly, BrdU uptake was reduced in the presence of both siRNAs before or after insulin treatment, when compared to scrambled siRNA transfected cells treated with insulin (Fig. 3G). Collectively, these results provide evidence that clathrin heavy chain- and caveolin-1-dependent translocation of the IR to the nucleus is necessary for insulin-induced proliferation *in vitro*. The fact that there appeared to be a stepwise decrease in nuclear IR with knockdown of clathrin, then caveolin, then both (Fig. 3F), but a similar decrease in BrdU uptake under all three circumstances (Fig. 3G) may reflect that the actions of other RTKs may have been inhibited as well. To examine whether impaired IR translocation to the nucleus affects insulin-induced Ca^{2+} signals, cells were analyzed by time-lapse confocal microscopy in the presence of scrambled siRNA and each or both cla and cav siRNAs. Silencing of either protein caused a decrease in both nuclear and cytosolic Ca^{2+} signals. Both nuclear and cytosolic Ca^{2+} signals were further impaired after simultaneous cla and cav silencing when compared to scrambled siRNA transfected

cells (Fig. 4A, B and C). These results provide evidence that *cla* and *cav*-mediated translocation of the IR from the PM to the nucleus is required to initiate insulin-induced Ca^{2+} signals. To confirm the specificity of these effects for insulin's action as a mitogen, we examined Akt activation, a known cytosolic action of insulin and the IR (17). Silencing of either or both proteins had no effect on Akt phosphorylation when compared to scrambled siRNA transfected cells treated with insulin (Fig. 4D and E); this indicates that this metabolic effect of insulin does not depend on IR translocation to the nucleus whereas nuclear Ca^{2+} signals and cell proliferation do. Collectively, these results demonstrate that *cla* and *cav*-mediated translocation of IR from the PM to the nucleus regulates insulin-induced Ca^{2+} signals and cell proliferation.

Liver regeneration but not insulin's metabolic effects depend on nuclear InsP_3

In order to determine the physiological relevance of observations in SkHep-1 cells, BrdU uptake experiments were performed *in vivo*. Cell proliferation was measured in Holtzman rats after partial (70%) hepatectomy (PH), under nuclear (InsP_3 -Buffer-NLS) (Fig 5A) or cytosolic (InsP_3 -Buffer-NES) InsP_3 buffering conditions. Holtzman is an outbred stock of the widely used Sprague-Dawley rat strain and has been used in other liver regeneration studies as well (30). The level of infection was monitored by confocal imaging of liver sections to detect mRFP positive cells. InsP_3 -Buffer-NLS was efficiently delivered and expressed in nearly 100% of the hepatocyte nuclei (Fig 5A-insert). A comparable efficiency of infection was observed in InsP_3 -Buffer-NES animals (not shown). BrdU uptake was impaired in animals expressing InsP_3 -Buffer-NLS, compared to control hepatectomized (PH) animals (Fig. 5B). Of note, expression of InsP_3 -Buffer-NES did not significantly alter BrdU uptake when compared to control PH animals, although this value was significantly higher than in InsP_3 -Buffer-NLS animals (Fig. 5B). Additionally, liver/body weight ratio after PH was reduced in InsP_3 -Buffer-NLS animals when compared to sham or PH animals (Fig. 5C). InsP_3 -Buffer-NES animals had a smaller liver/body weight ratio when compared to sham animals, although this value was not significantly different from control PH animals. Indeed, IR levels in the nucleus were increased 24 hrs after PH in PH animals compared to sham animals, as evidenced by immunohistochemistry (Fig. 5D) and immunoblot (Supporting Fig. 2). The 24 hr time point was chosen because it is the time at which the rate of DNA synthesis reaches its peak in hepatocytes after partial hepatectomy (2). Positive proliferating cell nuclear antigen (PCNA) labeling in PH animals confirms that hepatocytes are undergoing cell proliferation under these conditions (Supporting Fig. 2). These results show that liver regeneration after PH depends on nuclear InsP_3 , and increased nuclear IR may contribute, at least in part to this process, in accordance with our observations *in vitro*.

To investigate whether either cytosolic or nuclear InsP_3 participate in insulin's metabolic actions, we analyzed blood glucose levels and liver glycogen content under control, nuclear (InsP_3 -Buffer-NLS) and cytosolic (InsP_3 -Buffer-NES) InsP_3 buffering. Using one-way ANOVA with Bonferroni post-tests, cytosolic but not nuclear InsP_3 buffering significantly reduced blood glucose levels (Fig. 5E) and increased liver glycogen content, compared to control animals (Fig. 5F). These results are consistent with the idea that nuclear InsP_3 mediates insulin's effects on liver regeneration, but is unrelated to insulin's metabolic actions.

DISCUSSION

The effects of Ca^{2+} on hepatocyte proliferation are closely related to the subcellular compartments where it is released. For instance, buffering mitochondrial Ca^{2+} inhibits apoptosis and accelerates liver regeneration on that basis (30). On the other hand, buffering

cytosolic Ca^{2+} retards liver regeneration and progression through the cell cycle after partial hepatectomy (PH) (31), although there are a number of mechanisms by which cytosolic Ca^{2+} can increase (10), and different sources of cytosolic Ca^{2+} may have different effects (32). Here, we found that liver regeneration and insulin-induced cell proliferation both depend on nuclear but not cytosolic InsP_3 , which suggests that the only InsP_3 -mediated Ca^{2+} signaling that is relevant for liver regeneration occurs in the nucleus rather than the cytosol. Although buffering nuclear Ca^{2+} inhibits cell proliferation (16), inhibiting InsP_3 -mediated Ca^{2+} signals in the nucleus is more specific than solely buffering nuclear Ca^{2+} . Of note, because nuclear InsP_3 was buffered in our PH studies, this likely attenuated the proliferative effects not only of IR, but of c-met and EGFR as well. Therefore, these findings serve to provide the first *in vivo* evidence for the general importance of RTK-induced nuclear InsP_3 formation in liver regeneration.

Upon activation, the IR is internalized via endosomes. The acidic environment ($\text{pH} \sim 6$) within endosomes promotes ligand-receptor dissociation, as well as inactivation of the IR. Insulin is degraded within the endosome, and the IR is either sent to lysosomes for degradation or recycled to the plasma membrane for another round of binding, activation, and internalization (33). These trafficking steps are crucial for insulin signal transduction, since phosphorylated internalized receptors can bind to intracellular substrates in order to achieve insulin's biological actions (33). After insulin binding, the IR undergoes endocytosis through the classic clathrin-dependent pathway, as do other RTKs (28). It has been observed that a subpopulation of IR on the PM is associated with caveolin-enriched membrane domains (29, 34), although the functional significance of this has not been clear. Our results suggest that these endocytic pathways act together to mediate the translocation of the IR to the nucleus. This is consistent with the observation that clathrin is involved in EGFR nuclear translocation in SkHep-1 cells (12). Movement of plasma membrane receptors to the nucleus has also been described for other RTKs, for some G-protein-coupled receptors, and in various cell types (13, 15, 35, 36). The steps that follow endocytosis as the IR moves to the nucleus have not yet been elucidated. However, nuclear translocation of FGF is mediated by importin β (15) and movement of c-met to the nucleus depends on importin β and the chaperone GRB2-associated binding protein 1 (Gab1) (14), which can bind to other RTKs as well (37). However, whether these proteins also participate in IR translocation to the nucleus remains to be determined.

The metabolic actions of insulin in the liver are largely mediated by the phosphatidylinositol 3-kinase (PI3K)–Akt/protein kinase B (PKB) pathway. Akt is activated at the plasma membrane upon IR-mediated phosphorylation of PI3K (17). We found that Akt activation is not inhibited by blocking IR movement to the nucleus, suggesting that insulin's effects on cell proliferation are mediated by nuclear IR and are independent of IR in the cytosol. Interestingly, insulin's metabolic effects are enhanced in the liver-specific Gab1 knockout mouse (38). Because Gab1 may mediate nuclear translocation of RTKs (14, 37), the metabolic effects of insulin may be enhanced in the absence of Gab1 because of an increased amount of IR retained in the cytosol. In line with this hypothesis, our studies show that nuclear InsP_3 buffering decreased cell proliferation and liver weight after partial hepatectomy but did not affect insulin's metabolic actions, whereas cytosolic InsP_3 buffering had the opposite effects, suggesting that insulin's proliferative effects depend on nuclear InsP_3 , whereas its metabolic effects depend on cytosolic signaling events. Cytosolic InsP_3 buffering caused a decrease in blood glucose levels and an increase in liver glycogen content, although this might be due in part to inhibition of glucagon, which can increase cytosolic $\text{InsP}_3/\text{Ca}^{2+}$ (39).

Taken together, our observations provide evidence that insulin's effects on hepatocyte proliferation are due to nuclear and not cytosolic $\text{InsP}_3/\text{Ca}^{2+}$. Furthermore, nuclear InsP_3

formation is important for liver regeneration, although not only insulin but other growth factors such as HGF and EGF likely contribute to this process *in vivo*. Indeed, the signaling steps identified here, in which the IR trafficks from the PM to the nucleus to selectively and locally activate InsP₃/Ca²⁺ reveal a series of potential targets to regulate cell growth in the liver, to likely enhance cell proliferation after resection/living donor transplant. Therefore, this nuclear signaling pathway for insulin has broad and widespread clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

IR	Insulin Receptor
Ca²⁺	Calcium
InsP3	inositol 1,4,5,-trisphosphate
EGF	Epidermal Growth Factor
HGF	Hepatocyte Growth Factor
RTK	receptor tyrosine kinase
SH2	<i>src</i> -homology 2 domain
PI3K	phosphatidylinositol 3-kinase
PLC	Phospholipase C
PIP2	phosphatidylinositol 4,5-bisphosphate
DAG	diacylglycerol
PKC	protein kinase C
FGF	Fibroblast Growth Factor
PM	plasma membrane
NLS	nuclear localization sequence
NES	nuclear exclusion signal
BrdU	bromodeoxyuridine
cl	clathrin heavy chain
cav	caveolin-1
PH	partial hepatectomy
Gab1	GRB2-associated binding protein 1
PKB	Akt/protein kinase B

CHC hepatocellular carcinoma**REFERENCES**

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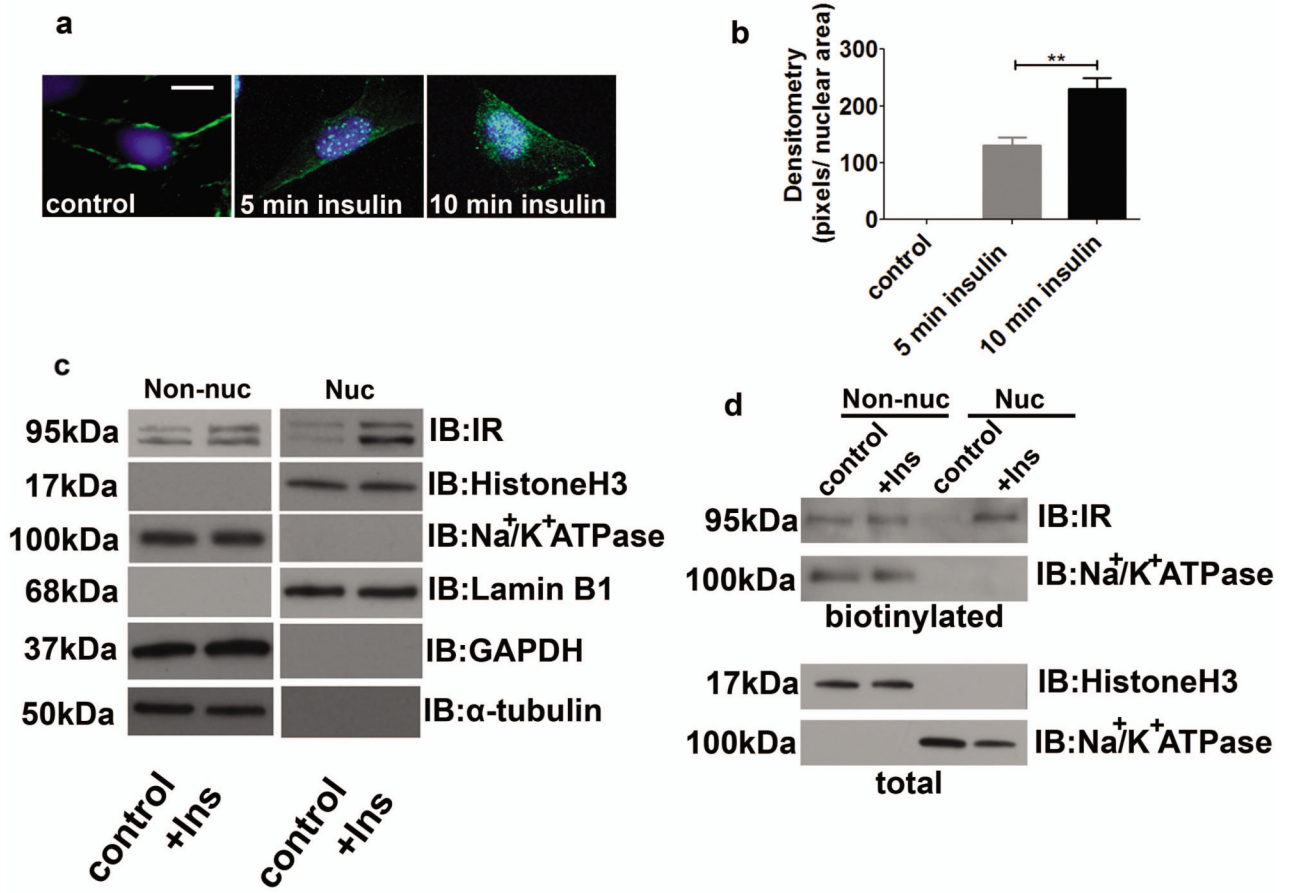


Figure 1. The insulin receptor translocates from the plasma membrane to the nucleus upon stimulation with insulin in SkHep-1 cells

A. Confocal immunofluorescence images of the insulin receptor before and after insulin stimulation (10 nM) for the indicated times in SkHep-1 cells. The insulin receptor was labeled in green and the nucleus was stained with dapi (blue). **B.** Quantification of nuclear fluorescence intensity in images shown in (A) (**p<0.001, n=3 experiments). Scale bar=10 μm. Values are mean ± S.D. Data were analyzed by one-way ANOVA followed by Bonferroni post-tests. **C.** Immunoblots of the insulin receptor (IR) in non-nuclear and nuclear fractions of SkHep-1 cells before and after insulin stimulation (10 nM). Histone H3 and Lamin B1 were used as purity controls for the nuclear fractions and Na⁺/K⁺ ATPase, GAPDH and α-tubulin for the non-nuclear fractions (n=3 experiments). **D.** Immunoblots of IR in biotinylated and fractionated SkHep-1 cells subjected to streptavidin pull-down before and after insulin (10 nM) stimulation. Na⁺/K⁺ ATPase was used to verify the purity of the non-nuclear and nuclear fractions as well as the biotinylated fractions; n=3 experiments.

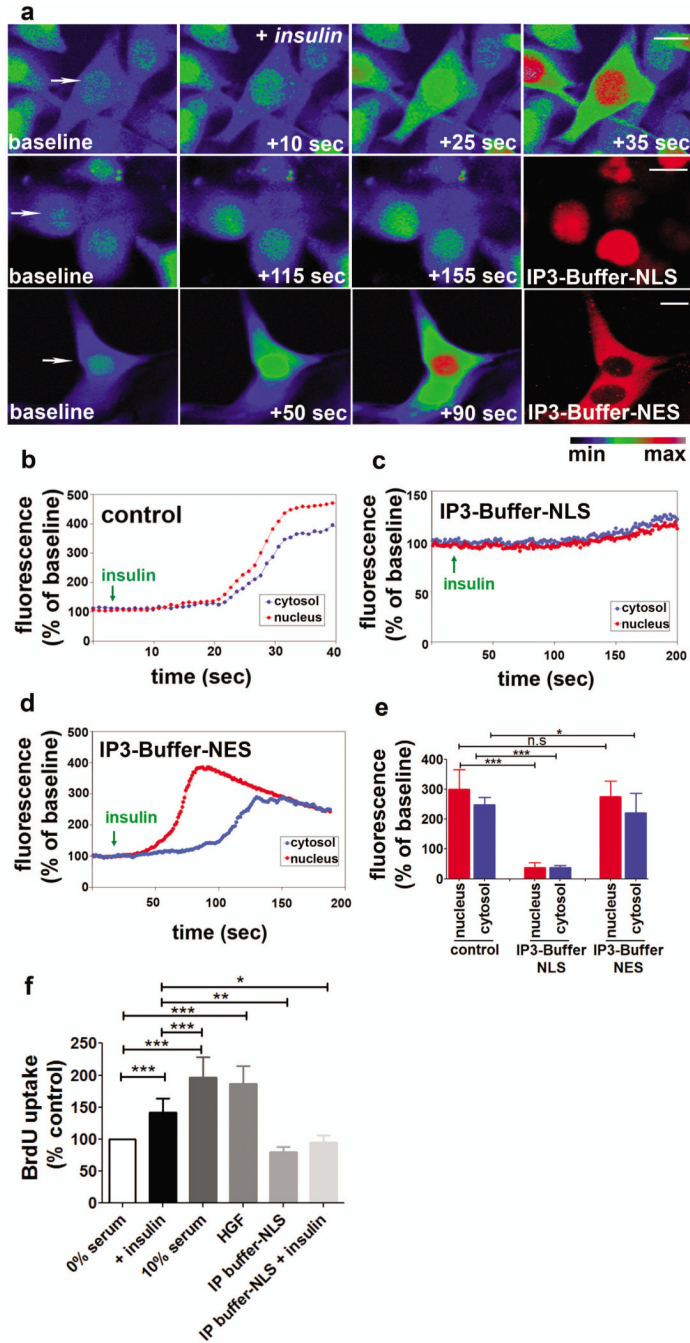
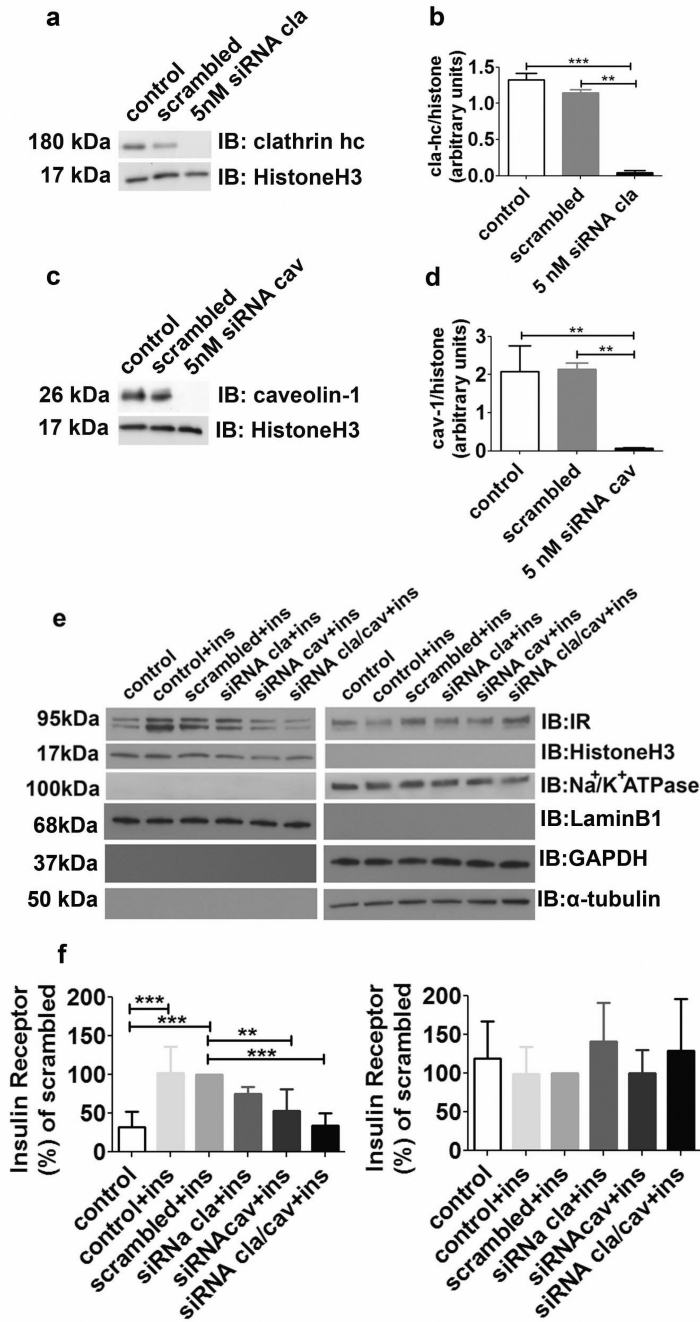


Figure 2. Insulin-induced Ca^{2+} signals and cell proliferation depend on nuclear $InsP_3$
A. Confocal images of SkHep-1 cells loaded with Fluo-4/AM (6 μ M) and stimulated with insulin (10 nM). Control, $InsP_3$ -Buffer-NLS and $InsP_3$ -Buffer-NES infected cells were analyzed. Right bottom panels show correct subcellular localization (red) of each adenoviral construct. Images were pseudocolored according to the scale shown at the bottom. Scale bar=10 μ m. Graphical representation of the fluorescence increase in the nucleus (red traces) and cytosol (blue traces) of control (**B**), $InsP_3$ -Buffer-NLS (**C**) and $InsP_3$ -Buffer-NES (**D**) cells. **E.** Summary of $InsP_3$ buffer studies. Values are mean \pm S.D of the peak Fluo-4 fluorescence acquired during the observation period (expressed as % of baseline) and include the response from 55 nontransfected cells, 55 cells expressing $InsP_3$ -Buffer-NLS,

and 44 cells expressing InsP₃-Buffer-NES (*p < 0.05, ***p<0.0001). **F.** BrdU uptake in control and InsP₃-Buffer-NLS infected cells before and after insulin (10 nM, 10 min) stimulation. 10% serum and HGF were used as additional positive controls for cell proliferation. (*p<0.05, **p<0.01, ***p<0.0001, n=3 experiments). Values are mean ± S.D Data were analyzed by one-way ANOVA followed by Bonferroni post-tests.



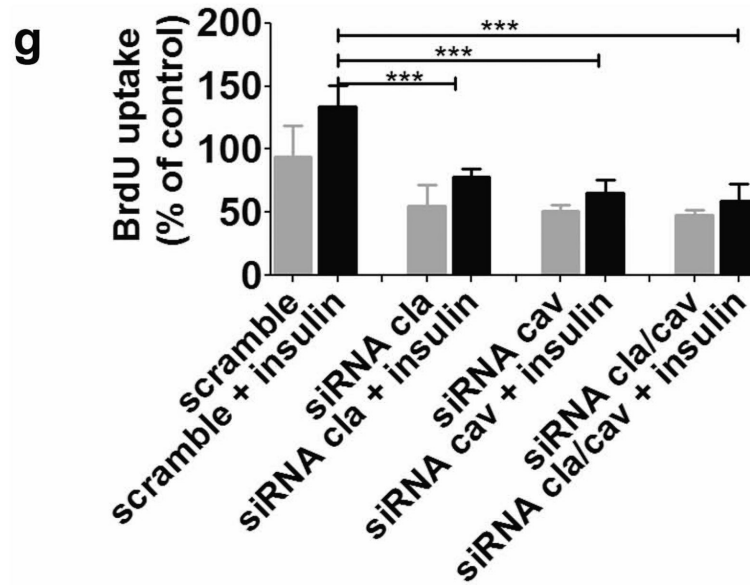


Figure 3. IR translocates to the nucleus in a clathrin heavy chain and caveolin-1-dependent manner to regulate insulin-induced cell proliferation

Immunoblots of total SkHep-1 cell lysates (**A,C**) and densitometric analysis (**B,D**) after treatment with 5 nM scrambled, clathrin heavy chain (**A,B**) or caveolin-1 (**C,D**) siRNAs. (** $p < 0.001$, *** $p < 0.0001$; $n = 3$ experiments). Values are mean \pm S.D. **E**. Immunoblots of non-nuclear and nuclear SkHep-1 fractions of cells stimulated with insulin (10 nM) under scrambled, clathrin heavy chain (cla), caveolin-1 (cav), and cla/cav silencing conditions. **F**. Densitometric analysis of (**E**). (** $p < 0.001$, *** $p < 0.0001$, $n = 5$ experiments). **G**. BrdU uptake in cells subjected to experimental conditions in (**E**). (* $p < 0.05$, *** $p < 0.0001$; $n = 3$ experiments). Values are mean \pm S.D. Data were analyzed by one-way ANOVA followed by Bonferroni post-tests.

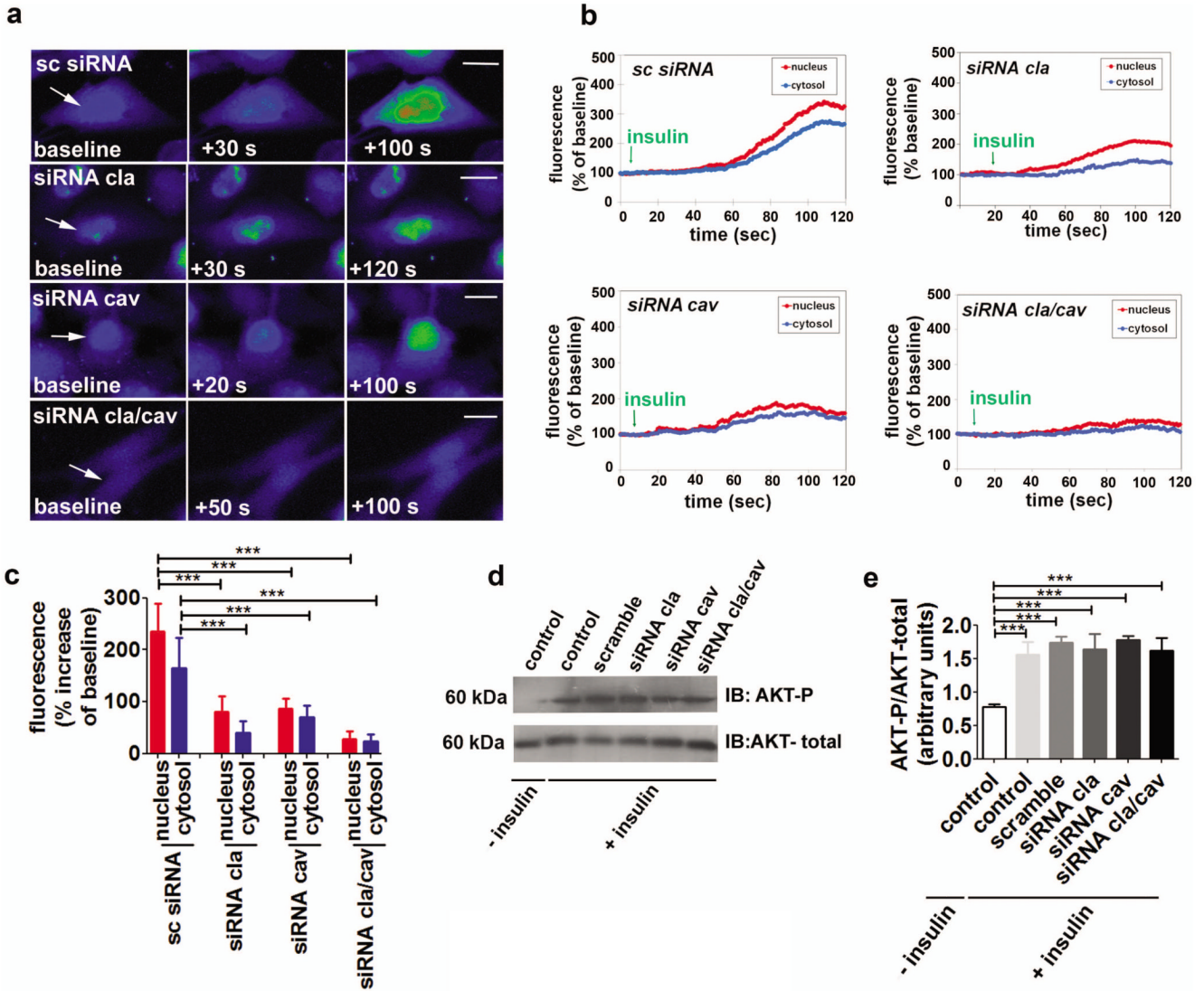


Figure 4. IR translocation to the nucleus mediates insulin-induced Ca²⁺ signals but not Akt activation

A. Confocal images of SkHep-1 cells loaded with Fluo-4/AM (6 μM) and stimulated with insulin (10 nM) under scrambled (sc), clathrin heavy chain (cla), caveolin-1 (cav) and cla/cav silencing conditions. Images were pseudocolored according to the scale shown at the bottom. Scale bar=10 μm. **B.** Graphical representation of the fluorescence increase in the nucleus (red traces) and cytosol (blue traces). **C.** Summary of siRNA studies. Values are mean ± S.D. of the peak Fluo-4 fluorescence acquired during the observation period (expressed as % of baseline) and include the response from 12 scrambled siRNA cells, 16 cla siRNA cells, 16 cav siRNA cells and 30 cla/cav siRNA cells (**p<0.0001). **(D)** Immunoblots of total SkHep-1 cell lysates. **(E)** Densitometric analysis of Akt phosphorylation in cells stimulated with insulin (10 nM) under scrambled, clathrin heavy chain (cla), caveolin-1 (cav), and cla/cav silencing conditions. (p<0.0001; n=3 experiments). Values are mean ± S.D. Data were analyzed by one-way ANOVA followed by Bonferroni post-tests.

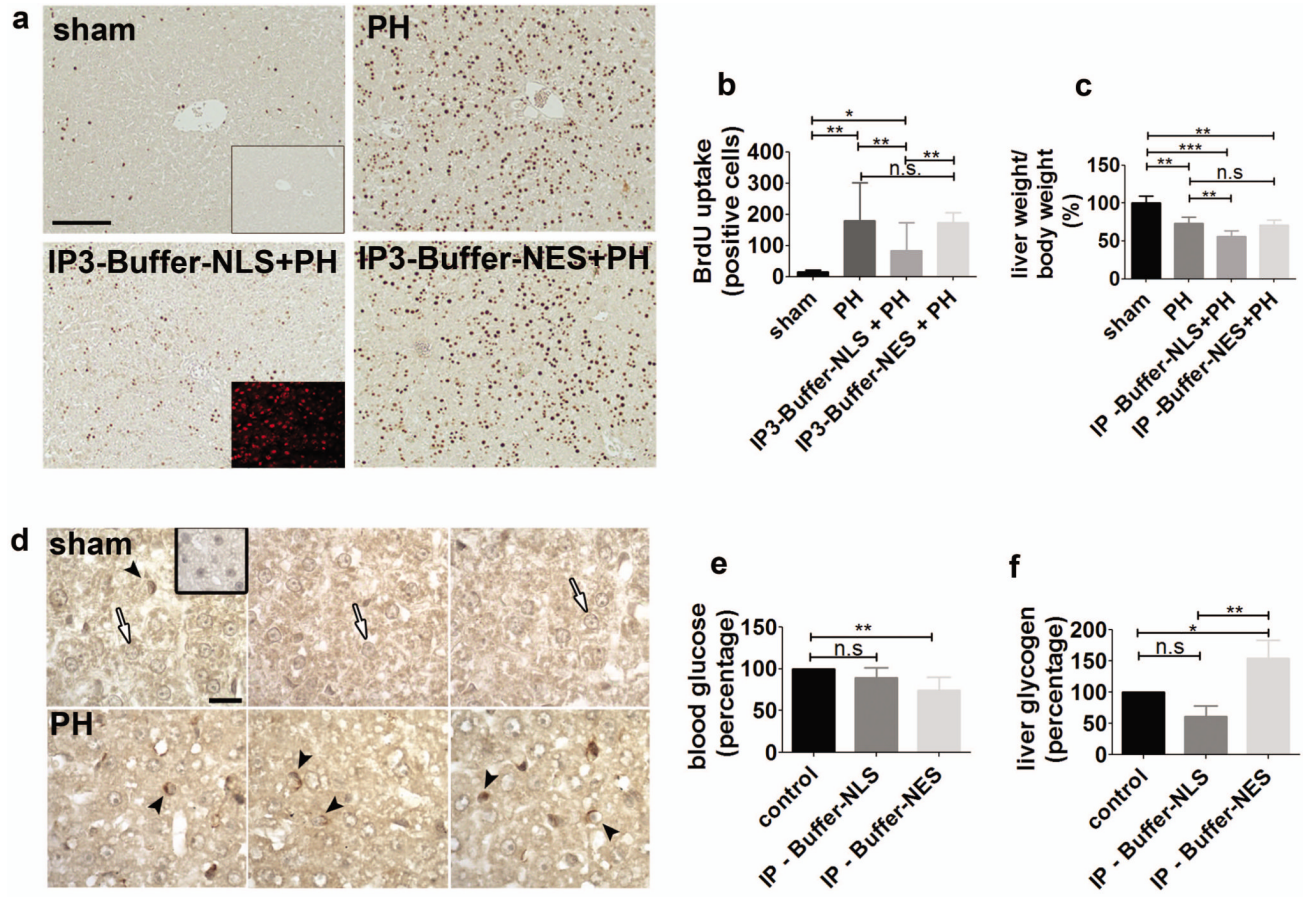


Figure 5. Liver regeneration but not insulin's metabolic effects depend on nuclear $InsP_3$

A. Immunohistochemistry images of liver sections from sham, PH, $InsP_3$ -Buffer-NLS and $InsP_3$ -Buffer-NES animals 48 hrs after PH and 72 hrs after infection and treatment with BrdU. BrdU staining in the nucleus allows the identification of proliferating cells in each group. Inset in sham panel represents the negative control of the technique. The near-100% efficiency of infection is highlighted in the insert in the $InsP_3$ -Buffer-NLS panel, in which a red nuclear stain is observed due to the mRFP tag. Scale bar=50 μ m. Objective lens: 10x. **B.** BrdU uptake in control non-hepatectomized (sham), control hepatectomized (PH) animals and PH animals (2 days after PH) infected with adenoviral forms of either $InsP_3$ -Buffer-NLS or $InsP_3$ -Buffer-NES for 72 hrs (* p <0.05, ** p <0.01; n =4 animals per condition). **C.** Liver/body weight ratio in animals subjected to experimental conditions in **A** (** p <0.001, *** p <0.0001; n =4 animals per condition). Values are mean \pm S.D. **D.** Immunohistochemistry images rarely detect the IR in the hepatocyte nucleus in sham (top row) but frequently detect it in PH animals (bottom row) 24 hrs after PH. Arrows: negatively stained nuclei; arrowheads: IR positive nuclei; insert: negative control; Scale Bar=40 μ m. Objective lens: 100x. **(E)** Quantification of blood glucose levels and **(F)** liver glycogen content in control animals, and in animals infected with either $InsP_3$ -Buffer-NLS or $InsP_3$ -Buffer-NES. (* p <0.05, ** p <0.001; n =4 animals per condition). Values are mean \pm S.D. Data were analyzed by one-way ANOVA followed by Bonferroni post-tests.