Cellular Signalling 28 (2016) 53–59

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

# Palmitic acid but not palmitoleic acid induces insulin resistance in a human endothelial cell line by decreasing SERCA pump expression

J. Gustavo Vazquez-Jimenez [a], Jesus Chavez-Reyes [a], Tatiana Romero-Garcia [a], Angel Zarain-Herzberg [b], Jesus Valdes-Flores [a], J. Manuel Galindo-Rosales [a], Angelica Rueda [a], Agustin Guerrero-Hernandez [a], J. Alberto Olivares-Reyes [a,*]

[a] Department of Biochemistry, Center for Research and Advanced Studies of the National Polytechnic Institute, CINVESTAV-IPN, Mexico DF 07360, Mexico
[b] Department of Biochemistry, School of Medicine, National Autonomous University of Mexico, DF 04510, Mexico

ARTICLE INFO

*Article history:*
Received 9 May 2015
Received in revised form 5 September 2015
Accepted 11 October 2015
Available online 22 October 2015

*Keywords:*
Insulin resistance
Endoplasmic reticulum stress
Palmitic acid
Palmitoleic acid
SERCA pump

ABSTRACT

Palmitic acid is a negative regulator of insulin activity. At the molecular level, palmitic acid reduces insulin stimulated Akt $Ser^{473}$ phosphorylation. Interestingly, we have found that incubation with palmitic acid of human umbilical vein endothelial cells induced a biphasic effect, an initial transient elevation followed by a sustained reduction of SERCA pump protein levels. However, palmitic acid produced a sustained inhibition of SERCA pump ATPase activity. Insulin resistance state appeared before there was a significant reduction of SERCA2 expression. The mechanism by which palmitic acid impairs insulin signaling may involve endoplasmic reticulum stress, because this fatty acid induced activation of both PERK, an ER stress marker, and JNK, a kinase associated with insulin resistance. None of these effects were observed by incubating HUVEC-CS cells with palmitoleic acid. Importantly, SERCA2 overexpression decreased the palmitic acid-induced insulin resistance state. All these results suggest that SERCA pump might be the target of palmitic acid to induce the insulin resistance state in a human vascular endothelial cell line. Importantly, these data suggest that HUVEC-CS cells respond to palmitic acid-exposure with a compensatory overexpression of SERCA pump within the first hour, which eventually fades out and insulin resistance prevails.



## 1. Introduction

Insulin resistance is the main condition leading to type 2 diabetes [1,2]. Obesity is a key factor associated with insulin resistance. Obese and diabetic humans have elevated plasma levels of non-esterified fatty acids (FFA), whereas palmitic acid (PA), the dominant saturated FA, is known to decrease insulin signaling [3].

The endoplasmic reticulum (ER) is a specialized organelle responsible for synthesis, folding and assembly of secretory and membrane proteins [4]. The ER needs a high luminal calcium concentration to function properly. In this respect, the sarco/endoplasmic reticulum calcium ATPase (SERCA) plays a key role, since it is responsible for maintaining high levels of calcium in the ER. Several reports have shown that increased saturated fatty acids and altered lipid composition of the ER results in a decreased expression and activity of SERCA pump [5,6]. Perturbation of ER homeostasis leads to stress and activation of unfolded protein response (UPR) [7,8], which induces activation of PERK, an integral membrane protein of the ER [9]. Maladaptive ER stress leads to the activation of JNK, which has been suggested to be involved in decreasing the insulin signaling pathway, which in turn results in the corresponding insulin resistance condition [10].

The induction of insulin resistance by saturated FA in cells with an important role in glucose metabolism has been described [11–13]. However, those studies about the effect that saturated FA has on insulin resistance of endothelial cells are rather limited [14,15]. Thus, the primary goal of this work was to study whether SERCA protein plays any role in the insulin resistance state induced by PA in vascular endothelial cells.

We have found that incubation of HUVEC-CS with PA induced a biphasic effect, an initial fast elevation followed by a sustained reduction of SERCA pump protein levels; while the insulin resistance state was associated with the diminished expression of SERCA pump. Overexpression of SERCA pump reversed the effect of PA on insulin signaling, suggesting that SERCA levels play an important role in insulin resistance induced by PA in human vascular endothelial cells.

## 2. Materials and methods

### 2.1. Materials and antibodies

Dulbecco's modified Eagle's medium (DMEM), actinomycin D (Act-D), palmitic acid (PA), palmitoleic acid (PAO) and fatty acid-free bovine

*Abbreviations:* ER, endoplasmic reticulum; PA, palmitic acid; SERCA, sarco/endoplasmic reticulum calcium ATPase; UPR, unfolded protein response.

* Corresponding author at: Department of Biochemistry, CINVESTAV-IPN, Av. IPN 2508, Col. San Pedro Zacatenco, Mexico DF 07360, Mexico.
*E-mail address:* jolivare@cinvestav.mx (J. Alberto Olivares-Reyes).

http://dx.doi.org/10.1016/j.cellsig.2015.10.001
0898-6568/

serum albumin (FAFBSA) were from Sigma Aldrich; fetal bovine serum (FBS) was from ByProductos; Attachment Factor protein (AF) 1× and Lipofectamine 2000 were from Life Technologies; SP600125 was purchased from Santa Cruz Biotechnology. Antibodies were commercially acquired as follows: p-Akt ($Ser^{473}$) (sc-7985, Santa Cruz Biotechnology), Akt (sc-8312, Santa Cruz Biotechnology), Actin (sc-1616, Santa Cruz Biotechnology), p-JNK ($Thr^{183}/Tyr^{185}$) (sc-6264, Santa Cruz Biotechnology), JNK (sc571, Santa Cruz Biotechnology), p-PERK ($Thr^{980}$) (#3179, Cell Signaling), PERK (sc-13073, Santa Cruz Biotechnology), SERCA2 (IID8) (#MA3-910, Thermo Scientific Pierce).

### 2.2. Cell culture of HUVEC-CS

The human umbilical vein endothelial cells (HUVEC-CS) were purchased from American Type Culture Collection. HUVEC-CS were grown at 37 °C in a humidified atmosphere of 95% air, 5% $CO_2$, in DMEM supplemented with 20% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin in plastic P-100 dishes that had been pretreated with AF for 30 min. All experiments were carried out using HUVEC-CS cells between passages 2 and 13. For experiments, HUVEC-CS cells were subcultured in 6-well plates (that had been pretreated with AF for 30 min) until cells have reached 80% confluence and then cultured with serum-free DMEM for another 6 h before PA or PAO treatments.

### 2.3. Lipid preincubations

Free fatty acid stock solutions at 500 mM (PA or PAO) were prepared in DMSO. HUVEC-CS cells were switched to serum-free DMEM with 1% FAFBSA followed by treatment with different concentrations of PA or PAO for the times indicated. The incubation with FAFBSA alone did not have any effect on the insulin-induced Akt phosphorylation in this type of cells (Suppl. Fig S1). In those experiments using Act-D, cells were preincubated either with the indicated concentrations of the drug or vehicle (PBS) for 1 h before the lipid treatment. At the end of the incubation, cells were stimulated with 100 nM insulin for 10 min before harvesting. To this end, cells were placed on ice, media were aspirated, and cells were washed twice with ice-cold PBS and lysed with 100 μl of Laemmli sample buffer 1×. These cell lysates were frozen at −20 °C until analyzed by Western blot.

### 2.4. Immunoblot analysis

Cell lysates were thawed to room temperature and briefly sonicated, heated at 99 °C for 5 min, and centrifuged at 14,000 rpm for 5 min. The supernatant was electrophoresed on SDS-PAGE (8 or 10%) gels and transferred to PVDF nylon membranes. Blots were incubated overnight at 4 °C with primary antibodies and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with Millipore Immobilon western HRP substrate peroxide solution. When necessary, membranes were stripped for reuse with Restore Western blot stripping buffer (Thermo scientific) for 30 min and the complete removal of the primary antibody was ensured. Quantification of immunoblot films was carried out with ImageJ. Loading controls for phosphorylated proteins used the total amount of the corresponding protein. In the case of SERCA2 pump, the loading control was actin.

### 2.5. Cell transfections

HUVEC-CS were seeded at $2.5 \times 10^4$ cells/well in 6-well plates that had been pretreated for 30 min with AF, and cultured for 3 days. After this time, cells were transfected with pEF1/His-A-hSERCA2b (1 μg/well) in 5 μg/ml Lipofectamine 2000 and using 1 ml of complete culture medium for 6 h at 37 °C. This was followed by fresh medium change and culturing for another 18 h prior to initiating incubation with lipids as previously described. The full-length human SERCA2b cDNA clone (ID 5503508) in pCMV-SPORT6 vector was obtained from Invitrogen. The human SERCA2b expression construct was made in the pEF1/His-A vector from Life Technologies. Briefly, the pCMV-SPORT6-SERCA2b vector was digested with NcoI and EcoRV to remove the SERCA2b insert and separated by electrophoresis in 1% agarose gel and eluted using the Gene III kit. Then the NcoI restriction site was filled with dNTPs and DNApolI Klenow fragment, dephosphorylated using CIAP and ligated using T4 DNA ligase into the vector pEF1/His-A vector previously digested with EcoRV and dephosphorylated. The integrity of the construct was verified by DNA sequencing.

### 2.6. Cell lysate and total cell membrane preparation

HUVEC-CS lysates (80% cell confluence) were prepared by resuspending cells (P-100 plates) in 10 ml of ice-cold homogenization buffer with protease inhibitors (300 mM sucrose, 20 mM HEPES, 2 mM EGTA, 0.153 μM aprotinin, 0.5 mM benzamidine, 12 μM leupeptin, and 0.1 mM PMSF, at pH 7.2). Cells were disrupted by passing cell suspension 4 times through an insulin syringe. A pellet was obtained by centrifugation at 100,000 ×*g* for 35 min at 4 °C and resuspended in 0.5 ml homogenization buffer without EGTA and then protein concentration was determined [16].

### 2.7. Determination of ATPase activity of SERCA pump

ATP hydrolysis by thapsigargin (TG)-sensitive SERCA pump was determined as reported by Bartolommei et al. [17], with some modifications. Briefly, ~400 μg of protein of total cell membrane preparation was incubated in 1.1 ml (final volume) of buffer solution (80 mM KCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 mM sodium azide, 0.2 mM KEGTA, 25 mM MOPS, and 2 μM A23187 at pH 7.0 adjusted with TRIS) at 37 °C for 5 min before starting the reaction by the addition of 1 mM ATP (final concentration). Aliquots of 0.1 ml were taken at 45 min and were immediately added to 0.9 ml of Pi (inorganic phosphate) reacting solution (125 mM $H_2SO_4$, 0.5 mM ammonium-heptamolybdate tetrahydrate, 10 mM ascorbic acid, 40 μM potassium antimony (III) tartrate hydrate). The color was developed after 10 min and absorbance was measured at 850 nm (Beckman Spectrophotometer DU800). Control experiments were performed in the presence of 100 nM TG. The difference between total and TG-insensitive ATPase activities was considered as SERCA pump hydrolytic activity. The reactions were carried out in duplicate. The standard Pi curve between 0.625 and 40 nmol was prepared from an aqueous stock solution of 400 μM $KH_2PO_4$. The water used for the preparation of all solutions was produced by a purification system (MilliQ Advantage A10).

### 2.8. Statistical analysis

Average intensities from Western blot films were analyzed using one-way ANOVA with Dunnett's post-test using PRISM, version 6.0 (GraphPad Software, San Diego, CA, USA). In all cases, p value $< 0.05$ was considered to be statistically significant. These data were normalized using either the control or the insulin responses and the mean ± S.E.M. was plotted for at least three separate experiments. Figures show representative blots.

## 3. Results

### 3.1. PA but not PAO induced insulin resistance in HUVEC-CS cells

Both PA and PAO are β-oxidized by mitochondria. However, PA, but not PAO, produces insulin resistance [18,19]. A well-established cellular indicator of insulin resistance is the inhibition of insulin-induced Akt ($Ser^{473}$) phosphorylation [20]. Therefore, we have determined whether PA or PAO produces insulin resistance in HUVEC-CS cells. Incubation of

cells that have been in the absence of serum for 6 h with 100 nM insulin resulted in the phosphorylation of Akt at ($Ser^{473}$), an effect that began at 2 min and persisted for as long as 30 min (the longest time tested, Suppl. Fig. S2). The 10 min time period was selected to study insulin responsiveness in HUVEC-CS cells. Incubation of cells with different concentrations of PA, from 0.25 to 1 mM, for 24 h, inhibited insulin-induced Akt ($Ser^{473}$) phosphorylation (Fig. 1A). This effect cannot be explained by degradation of Akt protein since total Akt did not show any significant change (Suppl. Fig. S3). In contrast, incubation of cells with PAO did not induce insulin resistance, except only at the highest concentration tested (1 mM), which showed a partial reduction of the insulin-induced Akt phosphorylation (Fig. 1). These data corroborate that it is the saturated fatty acid (PA), but not the unsaturated fatty acid (PAO), the one inducing insulin resistance in HUVEC-CS endothelial cells.

### 3.2. PA induces a biphasic effect on SERCA expression

It has been shown that *ob*/*ob* mice with insulin resistance have reduced SERCA pump expression in liver cells [21]. To determine whether PA alters the expression of SERCA2 in HUVEC-CS cells, we have carried out a time course of SERCA2 expression for cells incubated with either 0.25 mM PA or 0.25 mM PAO. As shown in Fig. 2A, PA (closed symbols) induced an initial increase of SERCA2 expression after 1 h of saturated fatty acid treatment (295 ± 15% control; $p < 0.05$) followed by a steady reduction of SERCA2 expression. At 8 h, the average expression of SERCA2 pump was below that of control; however, this reduction was significant only at 24 h of PA incubation. By contrast, PAO did not induce any change on the SERCA2 protein expression levels (Fig. 2B). Incubation of HUVEC-CS cells with a high concentration of PA (1 mM) still produced the biphasic effect on the SERCA2 pump expression (Suppl. Fig. S4). However, in this case the degradative effect promoted by PA was more evident, to the point that the compensatory elevation seen at 1 h with 0.25 mM PA was not significant at 1 mM PA. Moreover, the reduction in the expression of SERCA2 pump was significant at 12 h with 1 mM PA, 12 h earlier than with 0.25 mM PA. Thus, PA is promoting SERCA2 degradation, while PAO is not stimulating degradation of this pump.

### 3.3. PA induces protein expression of SERCA as a compensatory mechanism for ER stress

Since we have found that PA induces a biphasic effect on the expression of SERCA2 pump characterized by an initial increase in the levels of SERCA2 protein; we decided to explore further whether this effect was due to PA activating SERCA2 transcription in HUVEC-CS cells. As shown in Fig. 3A, preincubation of cells for only 1 h with 10 μM Act-D inhibited the initial effect of PA on elevating SERCA2 expression. In addition, we evaluated the turnover rate of SERCA pump by incubating cells with Act-D for 2 h. This condition resulted in a significant reduction of SERCA2 protein levels in naive cells, suggesting that SERCA2 pump has a high turnover rate in HUVEC-CS cells that have been in the absence of serum for 6 h (Fig. 3B). To determine the effect of PA on SERCA pump activity, cells were incubated for either 1 or 24 h with 0.25 mM PA and total cell membranes were prepared followed by determination of TG-sensitive ATPase specific activity (Fig. 3C). PA reduced ATPase activity at 1 h when it is considered that cells had increased expression level of SERCA pump at this time point; and as expected, this inhibitory

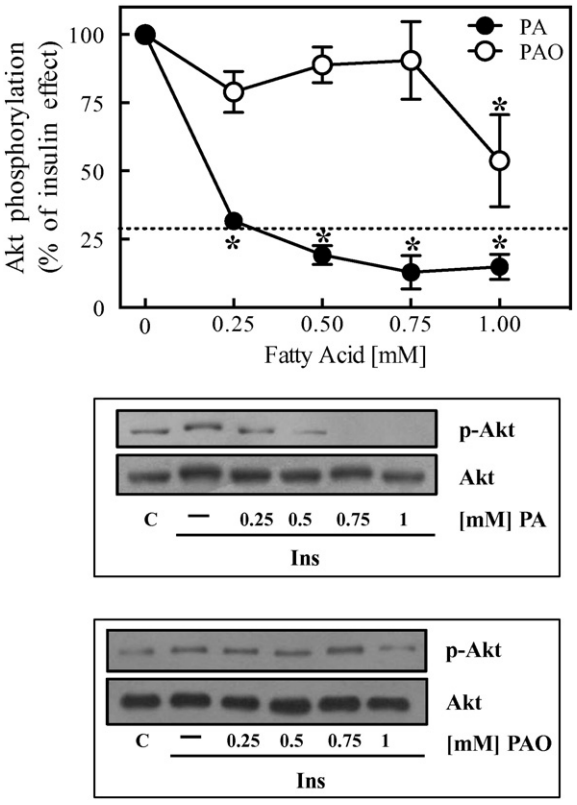


**Fig. 1.** Effect of PA and PAO on insulin signaling pathway. HUVEC-CS cells were preincubated for 24 h in control DMEM (with DMSO) or medium containing different concentrations of PA (solid circles) or PAO (open circles), followed by stimulation with 100 nM insulin for 10 min before being harvested. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-Akt ($Ser^{473}$). Quantification of 4–5 independent experiments are expressed as mean ± S.E. The dashed line indicates basal level of phospho-Akt. The lower panels show representative immunoblots. Western blots were also probed for total Akt as a loading control. *$p < 0.05$ *vs* insulin (−). Ins, insulin; PA, palmitic acid; PAO, palmitoleic acid.

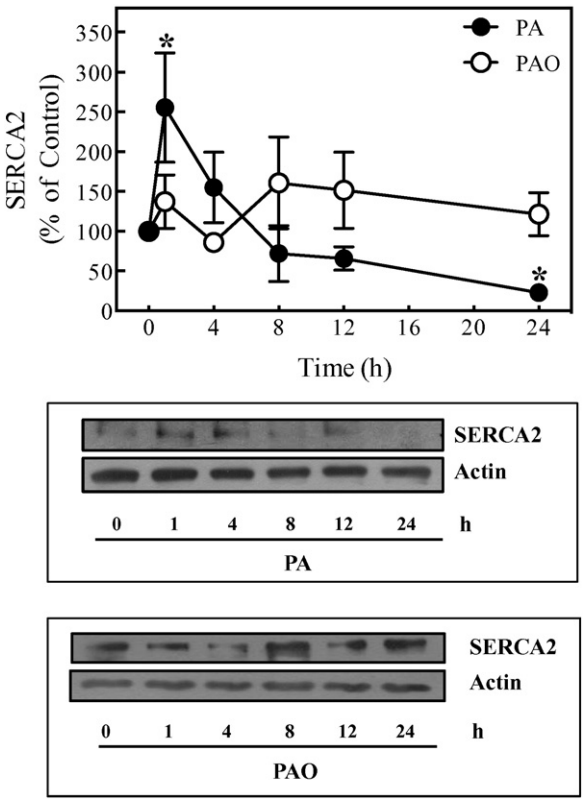


**Fig. 2.** Effect of PA and PAO on SERCA2 expression. HUVEC-CS cells were incubated with 0.25 mM PA (solid circles) or 0.25 mM PAO (open circles) at 37 °C for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-SERCA2. Quantification of 3 independent experiments are expressed as mean ± S.E. The lower panels show representative immunoblots. Western blots were also probed for actin as a loading control. *$p < 0.05$ *vs* 0 (control). PA, palmitic acid; PAO, palmitoleic acid.

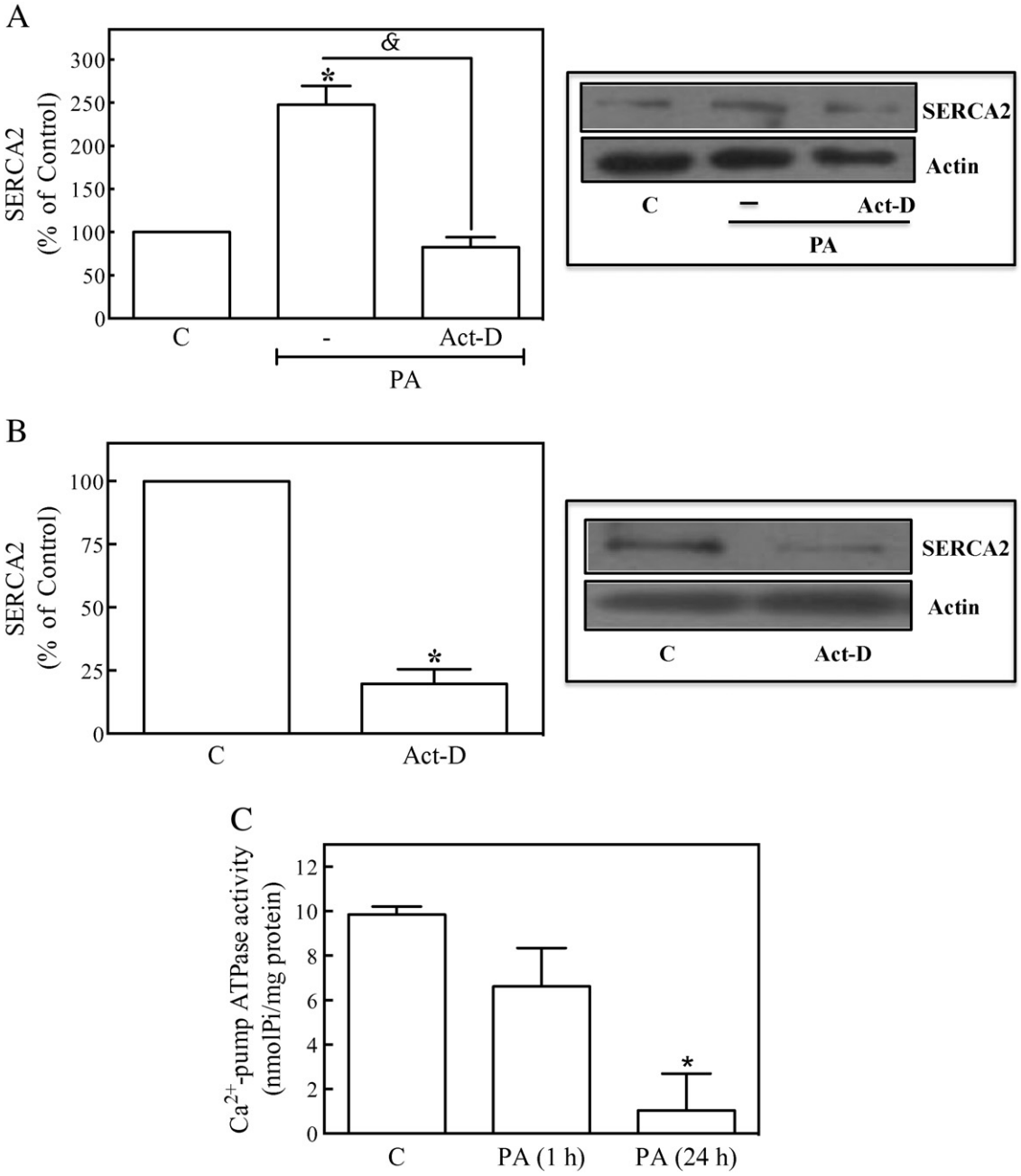


**Fig. 3.** Effect of transcriptional inhibition on PA-induced SERCA2 expression. (A) HUVEC-CS cells were preincubated in control medium (with PBS 2 h and DMSO 1 h) or medium containing 10 μM Act-D for 1 h followed by stimulation with 0.25 mM PA for 1 h before being harvested. (B) HUVEC-CS cells were preincubated in control medium (with PBS for 2 h) or medium containing 10 μM Act-D for 2 h. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-SERCA2. Quantification of 3–4 independent experiments are expressed as mean ± S.E. The panels on the right show representative immunoblots. Western blots were also probed for actin as a loading control. (C) HUVEC-CS cells were preincubated in control medium (with DMSO for 1 h) or medium containing 0.25 mM PA for 1 or 24 h before being harvested. Membrane fraction was used for measurement of TG-sensitive specific ATPase activity. Quantification of 4 independent experiments are expressed as mean ± S.E. (A) *$p < 0.05$ *vs* C and & $p < 0.05$ *vs* PA. (B) *$p < 0.05$ *vs* C. (C) *$p < 0.05$ *vs* C. Act-D, actinomycin D; C, control; PA, palmitic acid; TG, thapsigargin.

effect of PA on ATPase activity was very clear at 24 h when the protein level of SERCA pump was greatly diminished by PA. Collectively, these data suggest that saturated fatty acids promote early inhibition followed by SERCA2 degradation, which is initially compensated in HUVEC-CS cells by activating the transcription of this $Ca^{2+}$ pump.

### 3.4. PA induces ER stress before insulin resistance

It is well known that ER stress is involved in the induction of insulin resistance [9,10,22], and to determine whether PA is inducing ER stress in HUVEC-CS cells; these were incubated with PA at different times. PERK phosphorylation, which is indicative of ER stress (Fig. 4A), was evident with only 4 h of PA incubation. PERK phosphorylation is an adaptive response that tries to cope with ER stress; however, JNK activation indicates a maladaptive ER stress. JNK has been involved in the induction of the insulin resistance condition [9,23]. We have found that PA (0.25 mM) caused a strong phosphorylation of JNK at $Thr^{183}/Tyr^{185}$, which implies that JNK has been activated [3], reaching a maximum (~300%) at 8 h and sustained for another 4 h (Fig. 4B). Importantly, incubation of cells with PAO did not induce any ER stress, since neither PERK nor JNK were activated by this unsaturated fatty acid (Suppl. Fig. S5). Next, we have assessed what the state of the insulin- induced

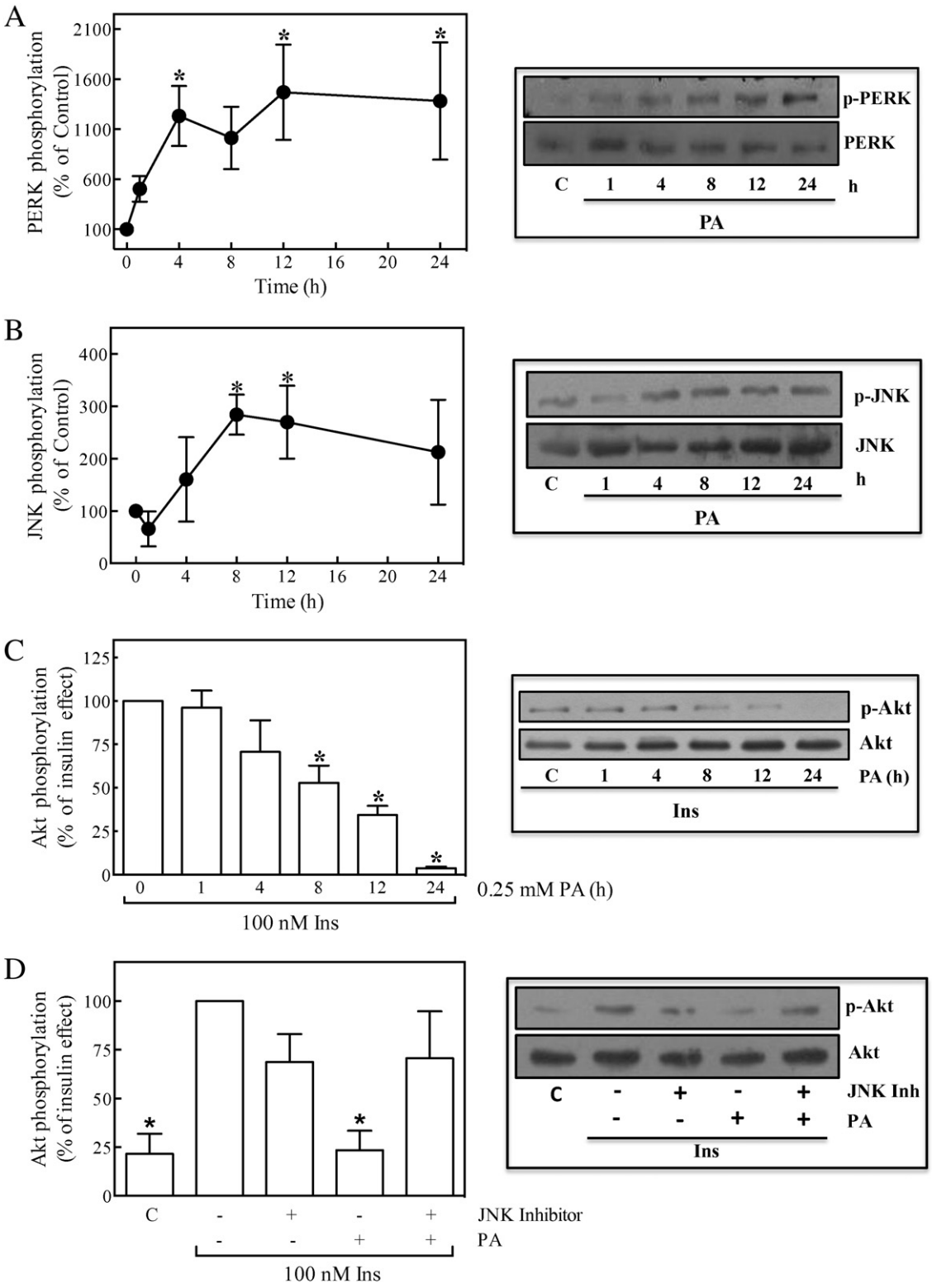


**Fig. 4.** PA induces ER stress and insulin resistance. HUVEC-CS cells were preincubated for 24 h in control medium (with DMSO) or medium containing 0.25 mM PA at different times, as indicated (A, B and C). (C) HUVEC-CS cells were preincubated for 24 h in control medium (with DMSO) or medium containing 0.25 mM PA at different times and stimulated with 100 nM insulin for 10 min before being harvested. (D) HUVEC-CS cells were preincubated for 1 h with or without SP600125 (JNK inhibitor) followed by incubation with or without PA for 8 h and stimulated with 100 nM insulin for 10 min, except the control. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-PERK ($Thr^{809}$) (A), anti-p-JNK ($Thr^{183}/Tyr^{185}$) (B) or anti-p-Akt ($Ser^{473}$) (C and D). The panels on the right show representative immunoblots. Quantification of 4–5 independent experiments are expressed as mean ± S.E. Western blots were also probed for total JNK (A), PERK (B) or Akt (C and D) to assess the total amount of these proteins. (A and B) *<p 0.05 *vs* 0 (control). (C) *p < 0.05 *vs* insulin. (D) *p < 0.05 *vs* insulin. Ins, insulin; PA, palmitic acid.

Akt phosphorylation was in these conditions. As shown in Fig. 4C, incubation of cells with 0.25 mM PA for 1 to 24 h, displayed a reduced insulin-induced Akt phosphorylation on $Ser^{473}$, an effect that we have shown cannot be explained by Akt degradation in response to PA (Suppl. Fig. S3). To corroborate the role of JNK in PA-induced insulin resistance, cells were preincubated with 1 μM JNK inhibitor (SP600125)

for 30 min before incubation with 0.25 mM PA for 8 h. The JNK inhibitor by itself did not have any effect on the insulin-induced Akt phosphorylation (Fig. 4D, third column) but this inhibitor restored insulin-induced Akt phosphorylation in the presence of PA (Fig. 4D, fifth column). Thus, these data suggest that PA might be desensitizing insulin signaling by a mechanism that involves ER stress and activation of JNK kinase [24].

### *3.5. Overexpression of SERCA in HUVEC-CS cells decreases insulin resistance induced by PA*

To determine the role played by SERCA2 pump in insulin resistance induced by PA in HUVEC-CS cells; we have transiently transfected these cells with the full-length human SERCA2b (pCMV-SPORT6/SERCA2b), which resulted in the increased expression of SERCA2 pump by 3-fold (see inset in Suppl. Fig. 6). As shown in Fig. 5, overexpression of SERCA pump reversed the inhibitory effect of 0.25 mM PA on insulin-induced Akt phosphorylation, while PA had the same inhibitory effect on Akt of mock cells. Interestingly, increasing the PA concentration to 0.5 mM reversed the protective effect of SERCA pump overexpression of insulin-induced Akt phosphorylation, although expression of SERCA2 was still elevated by the transfection (Suppl. Fig. S6). These results suggest that SERCA pump plays an important role in the state of insulin resistance induced by palmitic acid in endothelial HUVEC-CS cells. These data can be explained by assuming that PA, but no PAO, has increased rigidity of ER membranes, which results in the initial inhibition and followed later on by degradation of SERCA2 pump in HUVEC-CS cells.

## 4. Discussion

Previous studies have shown that saturated FAs induce insulin resistance in tissues with metabolic role [4,9,12,25,26]. It has been recently shown that high plasma concentrations of saturated FAs also impair endothelial cell function and associate with a number of increased cardiovascular risk factors that have been linked to insulin resistance; for instance, hypertension, dyslipidemia, and abnormal fibrinolysis [27,28]. However, the signaling pathways involved in the impairment of insulin signaling by saturated fatty acids in endothelial cells have not been completely characterized. Thus, in the present study, we have employed HUVEC-CS cells, as an endothelial cell model, because display well established endothelial cell characteristics [29], to investigate the effect of palmitic acid on insulin signaling. Our results show that PA inhibits insulin-induced Akt activation by a mechanism that involves alterations in SERCA2 expression and enzymatic activity; this should result in the loss of ER homeostasis, which then appears to be followed by maladaptive ER stress response (activation of JNK).

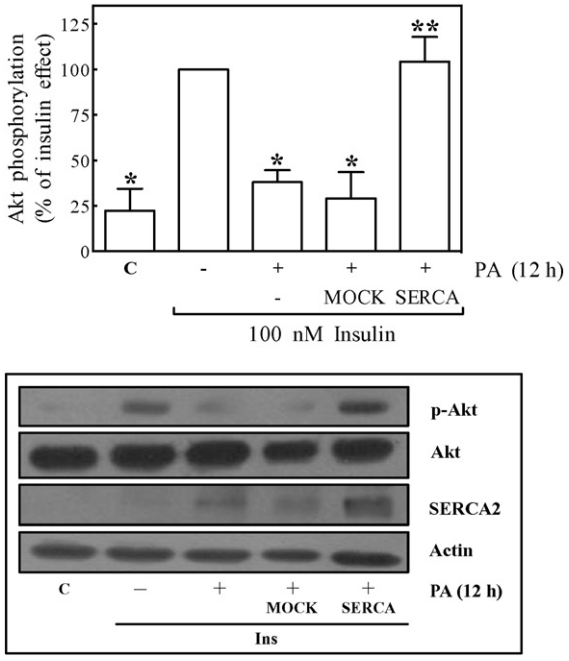


**Fig. 5.** SERCA2b overexpression decreases insulin resistance induced by PA. HUVEC-CS cells were preincubated in control medium (with Lipofectamine) or medium containing Lipofectamine with either mock plasmid or human SERCA2b expression plasmid for 6 h. After 24 h of transfection, HUVEC-CS cells were preincubated with vehicle (DMSO) or 0.25 mM PA for 12 h, followed by stimulation with 100 nM insulin for 10 min before being harvested. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-Akt ($Ser^{473}$) and anti-SERCA2. Lower panel shows representative immunoblots. Quantification of 5 independent experiments are expressed as mean ± S.E. Western blots were also probed for total Akt or actin. *$p < 0.05$ *vs* Ins. **$p < 0.05$ *vs* PA/MOCK. Ins, insulin; PA, palmitic acid.

Akt is an important downstream effector of insulin-mediated signaling in vascular endothelial cells, associated with increased blood flow that contributes significantly to insulin-mediated glucose uptake [30,31]. We observed in HUVEC-CS cells that insulin induced, as expected, phosphorylation of Akt at $Ser^{473}$. However, when cells have been exposed to PA, phosphorylation at this site was impaired. These results agree with those previously reported by Kim et al. [32], who demonstrated, in bovine aortic endothelial cells, that both insulin-mediated tyrosine-phosphorylation of IRS-1 and serine-phosphorylation of Akt, were significantly inhibited by treatment with PA. Importantly, this PA-mediated inhibition of insulin-induced Akt activation, was associated with the decrease of endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production, affecting insulin's physiological actions on the vasculature [32].

In view of recent data in rodents and humans implicating an important role of calcium homeostasis in the ER as a major contributor to the development of insulin resistance [33], we decided to study the molecular mechanism involved in the PA inhibitory effect on the insulin-signaling pathway in endothelial cells. Our data show that PA induces early changes in the SERCA2 pump expression. An initial increase followed by a sustained reduction that became significant at 24 h incubation with PA. In this context, there is a significant relationship between decreased SERCA expression and the state of insulin resistance in other types of cells. It has been shown that both obesity and diabetes condition are associated with a reduced SERCA2 pump expression [6,21,34]. However, it appears that this is not that straightforward. Induction of ER stress *in vivo* with either tunicamycin [7] or thapsigargin [8] modifies $Ca^{2+}$ signaling in heart cells that is corrected by expression of an active form of Akt kinase [7,8]. The case of thapsigargin is very interesting because this inhibitor of SERCA pump, produces ER stress by depleting $Ca^{2+}$ in the ER; however, the expression of an active form of Akt (that does not reduce ER stress markers) reveals that ER is replete with $Ca^{2+}$. This can be concluded because it is well known that heart $Ca^{2+}$ transient involves $Ca^{2+}$ release from ER *via* CICR. In any event, these works [7,8] show that the damaging effect of ER stress involves decreasing the activity of Akt and whether SERCA pump is upstream or downstream of Akt might depend on the experimental condition.

Interestingly, we observed that incubation of 0.25 mM PA for 1 h promotes increased SERCA2 expression (~2.5 folds). This SERCA expression may be associated with an initial compensatory response of the ER, an effect that has been previously reported by Caspersen et al., who demonstrated that ER stress inducers favored an increase in SERCA2 expression [35]. In addition, several independent lines of evidence support the up-regulation of SERCA2b mRNA as reflecting the activation of the UPR pathway [35]. In this context, our results show up-regulation of SERCA2 mRNA, based on the inhibition of PA-induced SERCA2 expression by actinomycin D. Remarkably, this increased SERCA2 expression was not able to prevent PERK activation, most likely due to PA decreasing the activity of even those newly synthesized SERCA2 pump. Along with the increase in SERCA expression as an indicator of ER stress, PERK phosphorylation was detected, which is also a *bona fide* indicator of ER stress [4,9,21,36,37]. This means that PA induces ER stress before

insulin resistance. Furthermore, maladaptive ER stress can induce the activation of JNK [3,9,23,38] that should lead to an impaired insulin-signaling pathway with a subsequent state of insulin resistance [9,26,38,39] and our results show that PA induces JNK phosphorylation while decreasing Akt phosphorylation (8 h) and a JNK inhibitor reverses the PA-induced insulin resistance condition.

One possible explanation for the biphasic response induced by PA on the SERCA2 expression would be that calcium transport activity by SERCA2 is compromised because of a loss in membrane fluidity caused by incorporation of PA in the ER phospholipids [40,41], which increases rigidity of the ER membrane. It has been shown that SERCA2 activity is decreased when the membrane order is increased [5], triggering the loss of ER $Ca^{2+}$ homeostasis, which should lead to the ER stress response. In this context, it has been shown that accumulation of free cholesterol and saturated FFA-containing phospholipids, but not unsaturated fatty acids, inhibits SERCA2b activity because of compositional changes in the ER phospholipids that can affect the membrane fluidity [5,42]. It has been demonstrated that alterations in ER fatty acid and lipid composition resulted in the inhibition of SERCA activity and ER stress. Interestingly, it has been shown that either correcting the obesity-induced alteration of ER phospholipid composition or inducing hepatic SERCA overexpression *in vivo*, both reduced chronic ER stress and improved glucose homeostasis [4,21].

There is evidence that thiazolidinediones, drugs that are prescribed to decrease insulin resistance, also promote the increase of SERCA2 expression [34,43,44]. Further, mice with insulin resistance restored sensitivity to this hormone in conditions that lead to overexpression of SERCA2 pump [21]. The fact that SERCA2b overexpression in HUVEC-CS cells decreases insulin resistance induced by PA at concentrations of 0.25 mM but not at 0.5 mM, indicates that SERCA is one of the first proteins that are affected by PA. In this sense it has been observed that PA produces a slow transient reduction of the ER luminal [$Ca^{2+}$] by 30 min, which is restored after 1 h [45]. While prolonged incubations with PA (24 or 48 h) increases the number of cells with depleted ER $Ca^{2+}$ levels [46].

In conclusion, our findings indicate that PA impairs insulin-induced Akt ($Ser^{473}$) by inhibiting the activity and expression of SERCA2 that leads to ER stress and the consequent activation of JNK. Once this kinase is activated it can block the insulin-signaling pathway resulting in a state of insulin resistance. These findings provide evidence that defines a role for SERCA2 pump and ER stress in the development of insulin resistance by PA in vascular endothelial cells.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

This research was supported by the Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional and partially by CONACYT/SEP Research Grants 167673 (to J.A.O-R.), 128735 (to A.G-H.), 127557, 236104 (to J.V-F.) and 164413 (to A.Z-H.); by CONACYT Ph. D. and MSc fellowship grants, No. 233232 (to J.G.V-J) and 554931 (to T.R-G), respectively. The authors thank Norma Cirnes for her help in the preparation of the figures, and Judith Hernandez-Aranda for technical help.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2015.10.001.

## References

[1] E. Chiefari, S. Tanyolac, F. Paonessa, C.R. Pullinger, C. Capula, S. Iiritano, T. Mazza, M. Forlin, A. Fusco, V. Durlach, A. Durlach, M.J. Malloy, J.P. Kane, S.W. Heiner, M. Filocamo, D.P. Foti, I.D. Goldfine, A. Brunetti, J. Am. Med. Assoc. 305 (2011) 903–912.
[2] J. Boucher, A. Kleinridders, C.R. Kahn, Cold Spring Harb. Perspect. Biol. 6 (2014).
[3] C.M. Mayer, D.D. Belsham, Endocrinology 151 (2010) 576–585.
[4] A.F. Ceylan-Isik, N. Sreejayan, J. Ren, J. Mol. Cell. Cardiol. 50 (2011) 107–116.
[5] Y. Li, M. Ge, L. Ciani, G. Kuriakose, E.J. Westover, M. Dura, D.F. Covey, J.H. Freed, F.R. Maxfield, J. Lytton, I. Tabas, J. Biol. Chem. 279 (2004) 37030–37039.
[6] S. Fu, L. Yang, P. Li, O. Hofmann, L. Dicker, W. Hide, X. Lin, S.M. Watkins, A.R. Ivanov, G.S. Hotamisligil, Nature 473 (2011) 528–531.
[7] Y. Zhang, Z. Xia, K.H. La cour, J. Ren, Antioxid. Redox Signal. 15 (2011) 2407–2424.
[8] Y. Zhang, J. Ren, Free Radic. Biol. Med. 51 (2011) 2172–2184.
[9] U. Ozcan, Q. Cao, E. Yilmaz, A.H. Lee, N.N. Iwakoshi, E. Ozdelen, G. Tuncman, C. Gorgun, L.H. Glimcher, G.S. Hotamisligil, Science 306 (2004) 457–461.
[10] S.J. Marciniak, D. Ron, Physiol. Rev. 86 (2006) 1133–1149.
[11] M.J. Park, D.I. Kim, J.H. Choi, Y.R. Heo, S.H. Park, Cell. Signal. 27 (2015) 1831–1839.
[12] T. Coll, E. Eyre, R. Rodriguez-Calvo, X. Palomer, R.M. Sanchez, M. Merlos, J.C. Laguna, M. Vazquez-Carrera, J. Biol. Chem. 283 (2008) 11107–11116.
[13] B.C. Lee, J. Lee, Biochim. Biophys. Acta 1842 (2014) 446–462.
[14] W.X. Guo, Q.D. Yang, Y.H. Liu, X.Y. Xie, M. Wang, R.C. Niu, Arch. Med. Res. 39 (2008) 434–442.
[15] Y. Lu, J. Cheng, L. Chen, C. Li, G. Chen, L. Gui, B. Shen, Q. Zhang, Biochem. Biophys. Res. Commun. 458 (2015) 1–7.
[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[17] G. Bartolommei, M.R. Moncelli, Tadini-Buoninsegni F, PLoS ONE 8 (2013), e58615.
[18] M. van Weeghel, H. te Brinke, H. van Lenthe, W. Kulik, P.E. Minkler, M.S. Stoll, J.O. Sass, U. Janssen, W. Stoffel, K.O. Schwab, R.J. Wanders, C.L. Hoppel, S.M. Houten, FASEB J. 26 (2012) 4316–4326.
[19] N.A. Talbot, C.P. Wheeler-Jones, M.E. Cleasby, Mol. Cell. Endocrinol. 393 (2014) 129–142.
[20] D.D. Sarbassov, D.A. Guertin, S.M. Ali, D.M. Sabatini, Science 307 (2005) 1098–1101.
[21] S.W. Park, Y. Zhou, J. Lee, J. Lee, U. Ozcan, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 19320–19325.
[22] J. Rieusset, M.A. Chauvin, A. Durand, A. Bravard, F. Laugerette, M.C. Michalski, H. Vidal, Biochem. Biophys. Res. Commun. 417 (2012) 439–445.
[23] Haberzettl P, Hill BG, Redox Biol. 2013;1:56–64.
[24] J. Hirosumi, G. Tuncman, L. Chang, C.Z. Gorgun, K.T. Uysal, K. Maeda, M. Karin, G.S. Hotamisligil, Nature 420 (2002) 333–336.
[25] S. Nakamura, T. Takamura, N. Matsuzawa-Nagata, H. Takayama, H. Misu, H. Noda, S. Nabemoto, S. Kurita, T. Ota, H. Ando, K.-I. Miyamoto, S. Kaneko, J. Biol. Chem. 284 (2009) 14809–14818.
[26] J.Y. Lee, H.K. Cho, Y.H. Kwon, Metabolism 59 (2010) 927–934.
[27] C. de Luca, J.M. Olefsky, FEBS Lett. 582 (2008) 97–105.
[28] B. Mlinar, J. Marc, A. Janez, M. Pfeifer, Clin. Chim. Acta 375 (2007) 20–35.
[29] S. Gifford, M. Grummer, S. Pierre, J. Austin, J. Zheng, I. Bird, J. Endocrinol. 182 (2004) 485–499.
[30] I. Hers, E.E. Vincent, J.M. Tavaré, Cell. Signal. 23 (2011) 1515–1527.
[31] G. Zeng, M.J. Quon, J. Clin. Investig. 98 (1996) 894–898.
[32] F. Kim, K.A. Tysseling, J. Rice, M. Pham, L. Haji, B.M. Gallis, A.S. Baas, P. Paramsothy, C.M. Giachelli, M.A. Corson, E.W. Raines, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 989–994.
[33] A. Guerrero-Hernandez, D. Leon-Aparicio, J. Chavez-Reyes, J.A. Olivares-Reyes, S. DeJesus, Cell Calcium 56 (2014) 311–322.
[34] V. Randriamboavonjy, F. Pistrosch, B. Bolck, R.H. Schwinger, M. Dixit, K. Badenhoop, R.A. Cohen, R. Busse, I. Fleming, Circulation 117 (2008) 52–60.
[35] C. Caspersen, P.S. Pedersen, M. Treiman, J. Biol. Chem. 275 (2000) 22363–22372.
[36] A.M. Nivala, L. Reese, M. Frye, C.L. Gentile, M.J. Pagliassotti, Metabolism 62 (2013) 753–760.
[37] T. Tian, Y. Zhao, S. Nakajima, T. Huang, J. Yao, A.W. Paton, J.C. Paton, M. Kitamura, Biochem. Biophys. Res. Commun. 410 (2011) 852–858.
[38] J.H. Lim, H.J. Lee, M. Ho Jung, J. Song, Cell. Signal. 21 (2009) 169–177.
[39] M.R. Kandadi, M.K. Unnikrishnan, A.K. Warrier, M. Du, J. Ren, Sreejayan N, J. Inorg. Biochem. 105 (2011) 58–62.
[40] N.M. Borradaile, X. Han, J.D. Harp, S.E. Gale, D.S. Ory, J.E. Schaffer, J. Lipid Res. 47 (2006) 2726–2737.
[41] J. Deguil, L. Pineau, E.C. Rowland Snyder, S. Dupont, L. Beney, A. Gil, G. Frapper, T. Ferreira, Traffic 12 (2011) 349–362.
[42] M. Cnop, F. Foufelle, L.A. Velloso, Trends Mol. Med. 18 (2012) 59–68.
[43] R.D. Shah, F. Gonzales, E. Golez, D. Augustin, S. Caudillo, A. Abbott, J. Morello, P.M. McDonough, P.J. Paolini, H.E. Shubeita, Cell. Physiol. Biochem. 15 (2005) 41–50.
[44] T. Kono, G. Ahn, D.R. Moss, L. Gann, A. Zarain-Herzberg, Y. Nishiki, P.T. Fueger, T. Ogihara, C. Evans-Molina, Mol. Endocrinol. 26 (2012) 257–271.
[45] K.S. Gwiazda, T.L. Yang, Y. Lin, J.D. Johnson, Am. J. Physiol. Endocrinol. Metab. 296 (2009) E690–E701.
[46] T. Hara, J. Mahadevan, K. Kanekura, M. Hara, S. Lu, F. Urano, Endocrinology 155 (2014) 758–768.