ISSN 0006-2979, Biochemistry (Moscow), 2019, Vol. 84, No. 5, pp. 553-561. © Pleiades Publishing, Ltd., 2019.
Russian Text © I. S. Stafeev, S. S. Michurina, N. V. Podkuychenko, M. Y. Menshikov, Ye. V. Parfyonova, A. V. Vorotnikov, 2019, published in Biokhimiya, 2019, Vol. 84, No. 5, pp. 719-729.

Chemical Inducers of Obesity-Associated Metabolic Stress Activate Inflammation and Reduce Insulin Sensitivity in 3T3-L1 Adipocytes

I. S. Stafeev[1,2,a]*, S. S. Michurina[1,3], N. V. Podkuychenko[1,3], M. Y. Menshikov[1], Ye. V. Parfyonova[1,2#], and A. V. Vorotnikov[1,4,b#]*

[1]*Institute of Experimental Cardiology, National Medical Research Center of Cardiology, 121552 Moscow, Russia*
[2]*Lomonosov Moscow State University, Faculty of Fundamental Medicine, 117192 Moscow, Russia*
[3]*Lomonosov Moscow State University, Faculty of Biology, 119991 Moscow, Russia*
[4]*Lomonosov Moscow State University, Medical Center, 119991 Moscow, Russia*
[a]*e-mail: yuristafeev@gmail.com*
[b]*e-mail: a.vorotnikov@icloud.com*

Received October 8, 2018
Revised January 23, 2019
Accepted January 23, 2019

Abstract—Obesity is accompanied by dyslipidemia, hypoxia, endoplasmic reticulum (ER) stress, and inflammation, representing the major risk factor for the development of insulin resistance (IR) and type 2 diabetes. We modeled these conditions in cultured 3T3-L1 adipocytes and studied their effect on insulin signaling, glucose uptake, and inflammatory response via activation of stress-dependent JNK1/2 kinases. Decreased insulin-induced phosphorylation of the insulin cascade components IRS, Akt, and AS160 was observed under all tested conditions (lipid overloading of cells by palmitate, acute inflammation induced by bacterial lipopolysaccharide, hypoxia induced by Co^{2+}, and ER stress induced by brefeldin A). In all the cases, except the acute inflammation, glucose uptake by adipocytes was reduced, and the kinetics of JNK1/2 activation was bi-phasic exhibiting sustained activation for 24 h. By contrast, in acute inflammation, JNK1/2 phosphorylation increased transiently and returned to the basal level within 2-3 h of stimulation. These results suggest a critical role of sustained (latent) vs. transient (acute) inflammation in the induction of IR and impairment of glucose utilization by adipose tissue. The components of the inflammatory signaling can be promising targets in the development of new therapeutic approaches for preventing IR and type 2 diabetes.

DOI: 10.1134/S0006297919050092

Keywords: insulin resistance, type 2 diabetes, inflammation, glucose transport

Obesity is one of the key problems in the modern society [1]. Obesity considerably increases the risk of type 2 diabetes mellitus, metabolic syndrome, their cardiovascular complications, and several types of cancer. In Russia, type 2 diabetes is diagnosed in 2.8% of the population, i.e., about 4.2 million people. The number of patients who are overweight is growing fast both among adults and children [2, 3]. Yet, the Russian science is far behind the world one in the study of cellular and molecular mechanisms of metabolic disorders, including those associated with type 2 diabetes and obesity.

Insulin resistance (IR) is the early event in the development of type 2 diabetes. IR is defined as a loss of insulin sensitivity of target cells due to inability of insulin to induce their glucose uptake from blood [4]. This results in stable hyperglycemia manifested by an increased level of glycated hemoglobin A1C. Despite a substantial progress in pharmacological correction of these parameters in dia-

Abbreviations: Akt, protein kinase B; AS160, Akt substrate of 160 kDa; BrA, brefeldin A; ER, endoplasmic reticulum; FFA, free saturated fatty acid (palmitate in this work); GLUT4, glucose transporter type 4; HIF-1α, hypoxia-inducible factor 1α; IR, insulin resistance; IRS, insulin receptor substrate; JNK, c-Jun *N*-terminal kinase; LPS, bacterial lipopolysaccharide; mTORC2, mammalian target of rapamycin complex 2; PI3K, phosphoinositide 3-kinase; TBST, Tris buffer supplemented with 0.1% Tween 20.

# These authors contributed equally to this work.
* To whom correspondence should be addressed.

betes therapy [2], the problem of counteracting and preventing IR as the primary event in pathogenesis of type 2 diabetes remains virtually unresolved. This is largely due to insufficient understanding of the mechanisms of IR development in the key insulin-targeted cells – adipocytes, myocytes, and hepatocytes.

Cellular mechanisms of IR development are believed to be associated with impaired intracellular signaling induced by insulin inside cells, which leads to reduced translocation of the insulin-dependent glucose transporter GLUT4 to the plasma membrane. The insulin cascade involves activation of insulin receptor, phosphorylation of insulin receptor substrate (IRS) at Tyr612 residue, activation of phosphoinositide 3-kinase (PI3K) cascade and protein kinase B (Akt kinase) by double phosphorylation at Thr308 and Ser473 residues, and phosphorylation of AS160 (Akt substrate of 160 kDa) that regulates GLUT4 translocation to the membrane [5-7]. Akt is phosphorylated at Thr308 by the PI3K-dependent phosphoinositide-dependent kinase PDK1, whereas Ser473 in Akt is phosphorylated by TORC2 (mammalian target of rapamycin complex 2), whose activity, at least partially, depends on PI3K [6]. Activation of insulin cascade is impaired upon IRS phosphorylation at serine residues by various protein kinases, which switches off the signaling function of IRS [7, 8].

It has been long believed that different serine kinases that suppress IRS function and lead to IR are activated at different pathological states associated with obesity. Depending on tissue type, these conditions include dyslipidemia, inflammation, hypoxia, oxidative stress, nutrient overload, and endoplasmic reticulum (ER) stress [7, 9-12]. There have been attempts to attribute predisposed phosphorylation of individual IRS serine residues by specific kinases to these conditions [13-15]. They resulted in a rather mosaic pattern, suggesting the absence of strict specificity and the possibility of a more general mechanism of IR induction. The use of knocked-out animal models revealed critical contribution of the inflammatory kinases such as IKKβ/ε and JNK1/2 [16-19] and suggested that latent inflammation could be a common mechanism that combines the effects of various risk factors. Association of obesity with inflammation in adipose tissue is well documented [20-22], yet there is no consensus still as to which of these two factors is the cause or the consequence [23]. Since several possible cross-talk pathways exist between the inflammatory and insulin cascades [12], this question has not been solved. Nevertheless, anti-inflammatory therapy can recover the sensitivity to insulin both on the system level [24, 25], and in the model 3T3-L1 adipocytes [26].

In this study, we aimed to model the pathological, obesity-associated conditions and compare their effects on insulin signaling and glucose uptake in cultured 3T3-L1 adipocytes.

MATERIALS AND METHODS

Culture and differentiation of 3T3-L1 preadipocytes. Mouse 3T3-L1 preadipocytes were obtained from the American Type Culture Collection (ATCC) and cultured in high-glucose Dulbecco modified Eagle's medium (DMEM) (4.5 g/liter glucose, 110 mg/liter sodium pyruvate, 2 mM L-glutamine, $6 \cdot 10^4$ U/liter penicillin, $6 \cdot 10^4$ U/liter streptomycin) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA, or Biological Industries, Israel). Adipogenic differentiation was performed for 10 days as described elsewhere [26, 27]. Briefly, 3T3-L1 preadipocytes were grown for 2 days up to ~90% confluency and then for 1 day more in DMEM with 10% newborn calf serum (NBCS; Gibco, USA). On the day 3, the medium was replaced with DMEM with 10% FBS, 0.5 mM dexamethasone, 0.25 μM isobutyl methyl xanthine, 2 μM rosiglitazone, 1 μg/ml insulin (all reagents were from Sigma-Aldrich, USA). The medium was exchanged daily for the next 2-3 days. On the day 5, the medium was replaced with DMEM containing 10% FBS and 1 μg/ml insulin; on the day 7, the differentiated adipocytes were transferred into high-glucose DMEM with 10% FBS. The cells were used in the experiments on the days 10 to 14 or fixed with 4% formaldehyde and stained for 1 h with the lipophilic dye OilRedO (Merck Millipore, USA).

Modeling the insulin resistance (IR). *Lipid-induced IR* was achieved as described by She et al. [28] with modifications. Mature 3T3-L1 adipocytes were deprived of FBS for 24 h in DMEM with 0.5% BSA. Then, 0.3 mM conjugate of palmitic acid with BSA (FFA) prepared according to Svedberg et al. [29] was added to cells. The insulin cell sensitivity was determined after 24 h by stimulation of cells with 100 nM insulin for 20 min, and measurement of [^{3}H]2-deoxyglucose uptake and phosphorylation of the insulin cascade proteins as described below.

Inflammation was induced by treatment of mature 3T3-L1 adipocytes with 50 ng/ml LPS (*E. coli*, 0111:B4 serotype; Sigma) for 24 h in DMEM supplemented with 10% FBS. Then, the cells were deprived of FBS for 4 h in high-glucose DMEM, and their sensitivity to insulin was determined by mesuring activatory phosphorylation of the main insulin cascade components and insulin-stimulated [^{3}H]2-deoxyglucose uptake as described below.

ER stress was induced according to Citterio et al. [30] with some modifications. Mature 3T3-L1 adipocytes cultured in DMEM supplemented with 10% FBS were treated with 100 μM BrA for 24 h. The cells were then deprived of FBS for 4 h. The insulin cascade activity and the uptake of [^{3}H]2-deoxyglucose were measured then as described below.

Hypoxia was modeled according to Glassford et al. [31] by stabilizing transcription factor HIF-1α with bivalent cobalt ions. Mature 3T3-L1 adipocytes were treated

for 24 h with 100 μM $CoCl_2$ in high-glucose DMEM containing 10% FBS and then incubated for 4 h in serum-free high-glucose DMEM with 100 μM $CoCl_2$. Then the insulin cascade activity and [^{3}H]2-deoxyglucose uptake were measured as described below.

The inflammatory response dynamics. The cells were treated with FFA, LPS, BrA, or $CoCl_2$ for 30 min and 1, 2, 8, and 24 h. The inflammatory response kinetics were assessed by increased activatory phosphorylation of Thr183/Tyr185 residues in the activation loop of the stress-dependent kinases JNK1/2.

Immunoblotting. Immunoblotting was used to compare the activation of phosphorylation of the insulin cascade proteins and to assess the kinetics of JNK1/2 phosphorylation (inflammatory response) in the IR models. The cells were lysed on ice in RIPA (RadioImmunoPrecipitation Assay) buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 8.0) containing inhibitors of proteases (cOmplete Tablets EASYpack; Roche, Switzerland) and phosphatases (10 mM sodium glycerophosphate, 20 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate). The cell lysate proteins were resolved by SDS-PAGE according to Laemmli [32] and transferred onto polyvinylidene fluoride (PVDF) membranes (no less than 1 A·h). The membranes were blocked for 2 h in 5% fat-free milk (AppliChem, Germany) in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and successively incubated with the primary and secondary antibodies in 1% milk in TBST in dilutions suggested by the manufacturer.

Insulin cascade activation was determined by phosphorylation of IRS-1, Akt, and AS160. The used antibodies were against phospho-Tyr612 in IRS-1 (#44816; Thermo Fischer Scientific, USA), total IRS-1 (3407; Cell Signaling Technology, USA), phospho-Thr308 in Akt (9275; Cell Signaling Technology), phospho-Ser473 in Akt (4060; Cell Signaling Technology), total Akt (ab64148; Abcam, USA); phospho-Ser318 in AS160 (8619; Cell Signaling Technology), total AS160 (2670; Cell Signaling Technology), and vinculin (ab18058; Abcam). The secondary antibody was anti-rabbit IgG conjugated with horseradish peroxidase (ab6721; Abcam). JNK activation was assessed with phospho-specific antibodies against phospho-Thr183/phospho-Tyr185 in JNK1/2 (AF1205; R&D Systems, USA) and total anti-JNK1/2 antibodies (AF1387; R&D Systems).

The protein bands were visualized by chemiluminescence with Clarity ECL reagents (Bio-Rad, USA) using a FusionX gel-documentation system (Vilber Lourmat, France) in the cumulative video mode to control the linearity of the signal. For quantitative densitometry the GelAnalyzer2010a software was used. To determine the increase in phosphorylation level in response to insulin, we first determined the relative phosphorylation level of a protein of interest as the ratio of the chemiluminescence signal of the phosphorylated protein to that of the total protein. Then the increase in insulin-induced phosphorylation was calculated as the ratio of phosphorylation level in insulin-stimulated to that in non-stimulated cells.

Measurement of the insulin-induced [^{3}H]2-deoxyglucose uptake. Glucose uptake by adipocytes was measured after modeling IR conditions as above. The cells were deprived of FBS for 4 h in high-glucose DMEM with 0.1% BSA and washed three times with glucose-free DMEM; then insulin was added to final concentration of 100 nM for 20 min. To correct for insulin-independent glucose uptake, 20 μM cytochalasin B was added for 20 min to control cells. Then, the medium was replaced with the medium containing 100 μM 2-deoxyglucose + 0.5 μCi [^{3}H]2-deoxyglucose (PerkinElmer, USA). After 10 min, the cells were carefully washed twice with an ice-cold phosphate buffered saline (PBS) and frozen at −20°C to disrupt cell membranes. Then cells were thawed and lysed in 300 μl of RIPA buffer; 100 μl of the lysate was used to determine protein concentration with bicinchoninic acid (Pierce BCA Protein Assay Kit; ThermoScientific, USA). The remaining 200 μl of the lysate was dissolved in Beckman Ready-Solv HP scintillation fluid (Beckman, USA). The radioactivity was measured with a RackBeta counter (LKB Wallac, Sweden), and the results were normalized to the protein amount in samples. The cpm values obtained in the presence of cytochalasin B were subtracted from those obtained in its absence to eliminate contribution of the insulin-independent glucose transport.

Statistical analysis. The data were processed statistically with the MS Excel 2007 software. The results were presented in histograms as mean values ± standard deviation. The significance of differences was calculated using the paired *t*-test with different sample variance; the *p* values < 0.05 were considered statistically significant.

RESULTS

Chemical IR inducers reduce insulin cascade activation in 3T3-L1 adipocytes. IRS phosphorylation at Tyr612 is the initial step of insulin signaling toward PI3K [5]. Figure 1a shows that insulin-induced stimulation of Tyr612 phosphorylation in IRS was reduced after the treatment of mature 3T3-L1 adipocytes with each of the tested chemical inducers of IR. These results indicate that lipid overloading of cells, inflammation, hypoxia, and ER stress reduce insulin ability to activate signaling at the IRS level.

Signaling via the PI3K cascade results in the activation of the Akt kinase via phosphorylation at two residues, Thr308 and Ser473 [6]. All chemical inducers of IR reduced insulin-stimulated increase in Akt phosphoryla-

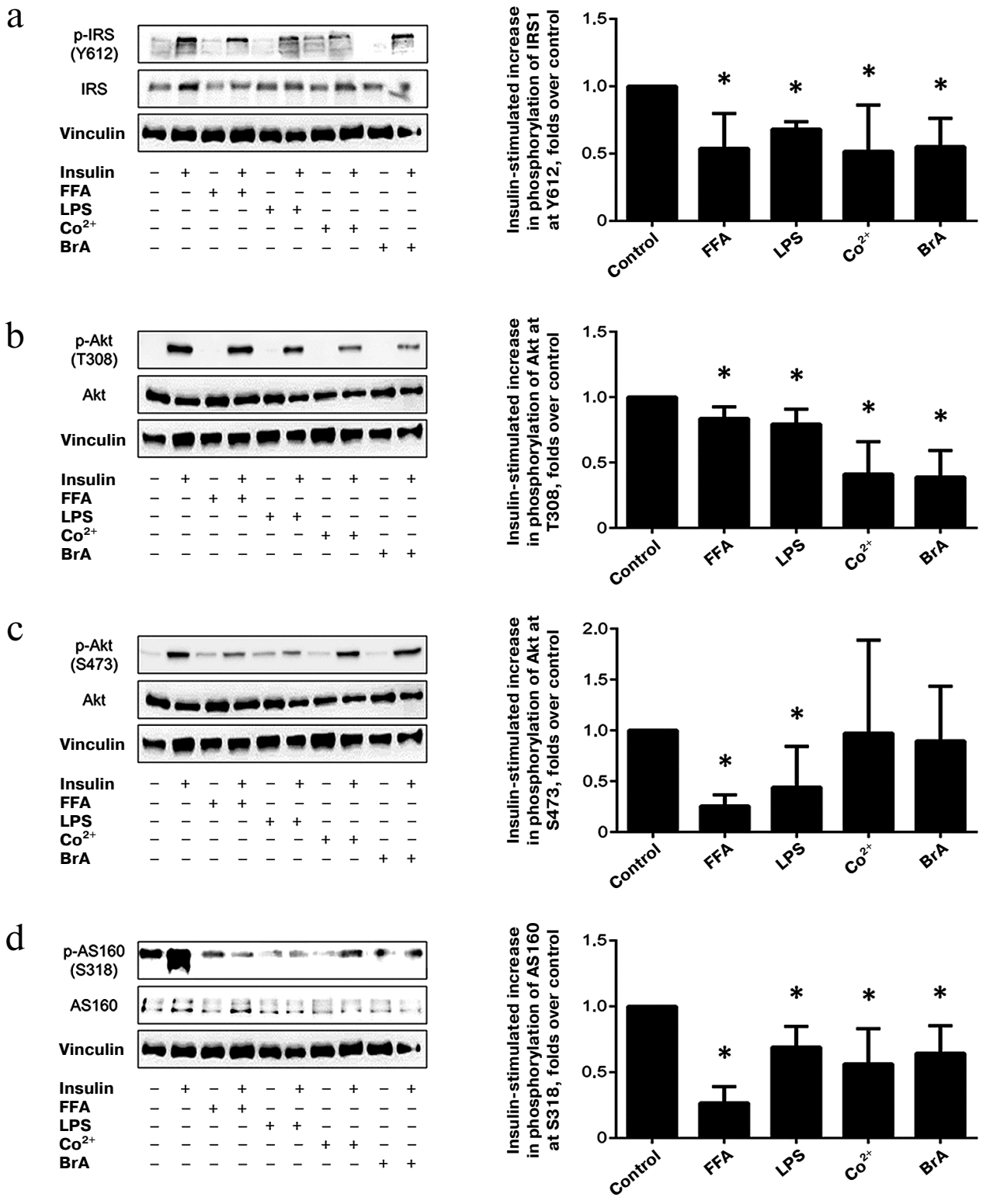

Fig. 1. Chemical IR inducers reduce insulin-dependent phosphorylation of the key insulin signaling components in 3T3-L1 adipocytes. a) Representative Western blots (left panel) and statistics (right panel) of IRS phosphorylation at Tyr612; b) representative Western blots (left panel) and statistics (right panel) of Akt phosphorylation at Thr308; c) representative Western blots (left panel) and statistics (right panel) of Akt phosphorylation at Ser473; d) representative Western blots (left panel) and statistics (right panel) of AS160 phosphorylation at Ser318. Results in the right panels are shown as mean ± standard deviation; * $p < 0.05$; $n = 3$.

tion to different extent (Fig. 1). While LPS and FFA weakly reduced it for Thr308 phosphorylation (Fig. 1b), cobalt ions and BrA decreased it 2-3-fold. The situation was opposite for Ser473: Co^{2+} and BrA had virtually no effect, whereas LPS and FFA decreased insulin-stimulated phosphorylation of Ser473 nearly 3-fold (Fig. 1c). However, because both the residues are needed for Akt activation, it suggests that all chemical inducers impair insulin-induced activation of the PI3K cascade and of Akt as its target.

To further confirm inactivation of insulin signaling toward GLUT4, we followed changes in insulin-dependent phosphorylation of AS160 at Ser318 residue. Akt is a direct regulator of AS160, targeting mainly its Ser318 [33, 34]. As shown in Fig. 1d, all chemical inducers of IR similarly reduced the insulin-stimulated increase in phosphorylation of AS160. Therefore, lipid overloading, inflammation, hypoxia, and ER stress impair Akt activation by insulin and subsequent signaling to GLUT4.

All chemical inducers of IR except LPS reduce insulin-stimulated [^{3}H]2-deoxyglucose uptake by 3T3-L1 adipocytes. The effect of insulin on glucose transport into adipocytes was measured by a routine approach using radio-labeled glucose analog. The contribution of insulin-independent glucose transporters was determined in the presence of cytochalasin B (an inhibitor of GLUT4 translocation to the plasma membrane). As shown in Fig. 2, all chemical IR inducers, except LPS, reduced the insulin-stimulated uptake of [^{3}H]2-deoxyglucose. This does not entirely correlate with the changes in the insulin cascade activity shown above. LPS did not affect the insulin-dependent uptake of deoxyglucose by the cells, despite it reduced activation of the insulin cascade (Fig. 1).

All chemical IR inducers except LPS cause prolonged activation of the stress-dependent JNK1/2 kinases in 3T3-L1 adipocytes. Stress-dependent kinases JNK1/2 are activated in obesity and play an important role in the IR development [12]. We have followed the kinetics of JNK1/2 activation in 3T3-L1 adipocytes during chemical induction of IR. Lipid overloading, inflammation, hypoxia, and ER stress promoted phosphorylation of the activating residues in JNK1/2; however, the kinetics of JNK1/2 activation under these conditions was different (Fig. 3).

The level of JNK1/2 phosphorylation quickly increased after addition of the inducers to the cells, which corresponded to the first, acute phase of the inflammatory response (1-2 h). However, further kinetics markedly differed for different inducers with respect to the presence or absence of the second, prolonged phase (3-24 h). While FFA and BrA stably increased the level of JNK1/2 phosphorylation, the second phase was virtually absent in the case of LPS. After treatment with Co^{2+}, the second phase was present, but the level of JNK1/2 phosphorylation decreased by 24 h of the treatment.

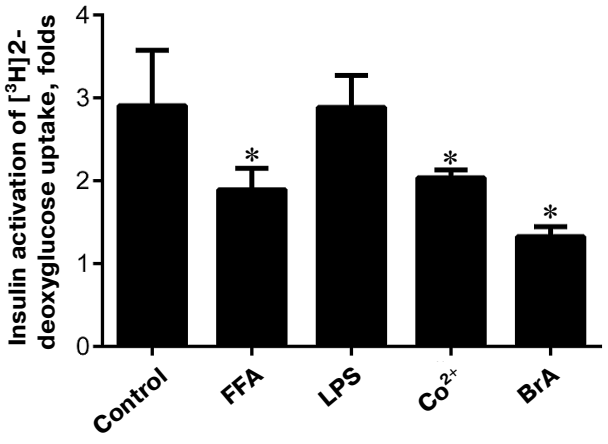

Fig. 2. Effect of chemical IR inducers on the insulin-stimulated increase in [^{3}H]2-deoxyglucose uptake by 3T3-L1 adipocytes. The data are presented as mean ± standard deviation; * $p < 0.05$, $n = 6$.

DISCUSSION

In this work, we tested the hypothesis that *in vitro* modeling of different pathological obesity-associated condition can induce IR and affect glucose transport into adipocytes. In all the cases (lipid overloading, acute inflammation, hypoxia, and ER stress), the activating effect of insulin was reduced at the (i) initial (IRS phosphorylation), (ii) intermediate (phosphorylation of Akt kinase as a target of the PI3K cascade), and (iii) final (phosphorylation of the insulin cascade effector AS160 that regulates activity of the insulin-dependent glucose transporter GLUT4) stages. Under all tested conditions, an increased phosphorylation of the stress-dependent kinases JNK1/2 (normally activated in inflammation) was observed; however, the kinetics of JNK1/2 activation was different and transient. Since JNK1/2 are known as the major serine kinases of IRS, our data suggest the existence of a common inflammatory mechanism responsible for the development of IR of various etiology.

Mechanisms of the insulin cascade activity reduction under the influence of FFA. It is well known that saturated free fatty acids (FFAs) suppress glucose uptake by the insulin-dependent tissues [35, 36]. This physiological phenomenon underlying the Randle glucose–fatty acid cycle [35] was described in detail for muscle cells as major consumers of circulating glucose in the organism. However, this cycle is much less studied in adipocytes where excessive glucose was thought to mainly suppress lipolysis. Only later, it was shown that the excess of FFAs (of dietary or lipolytic origin) also decreases glucose transport into adipocytes [37, 38]. Despite a great number of works confirming this phenomenon [36], its cellular mechanism for a long time remained unclear.

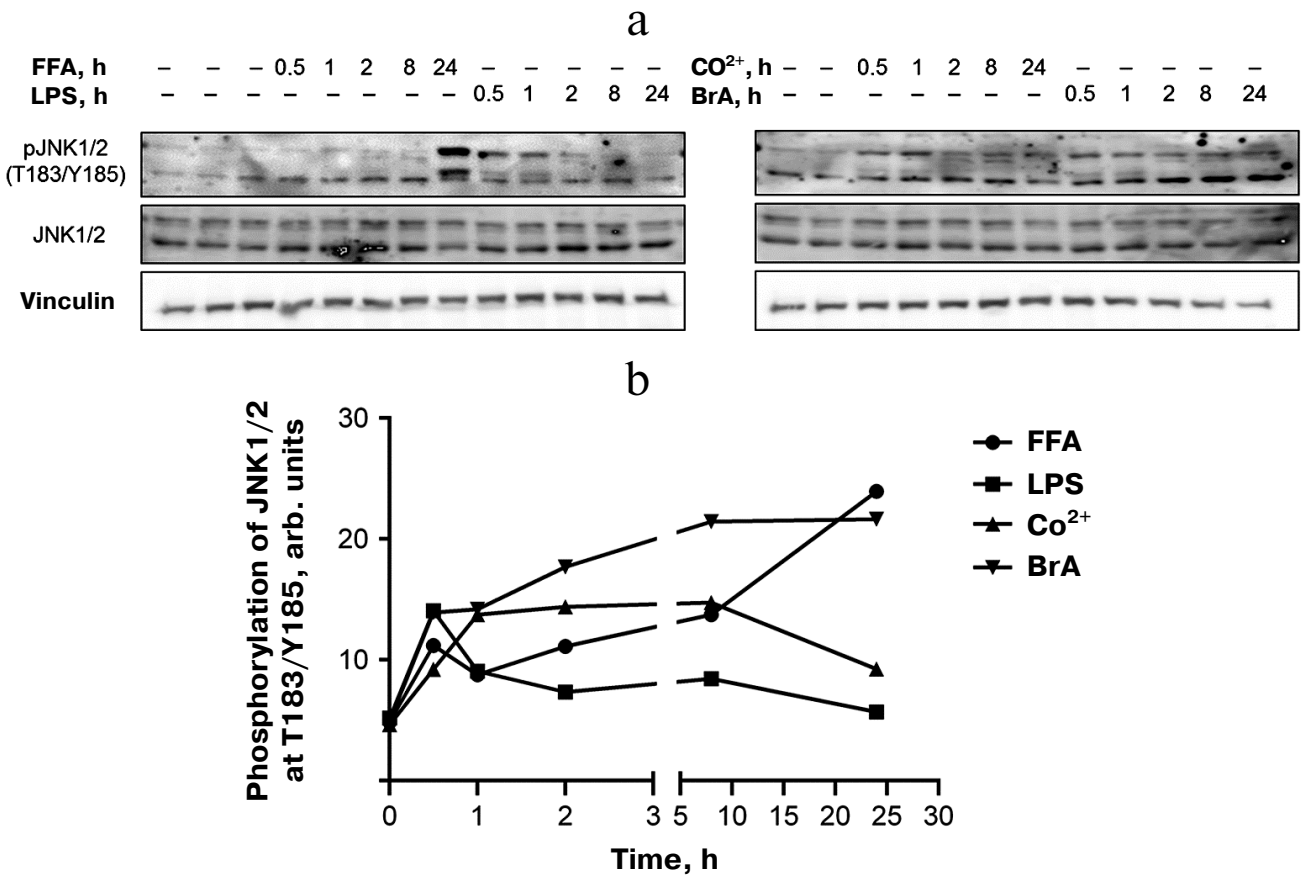

Fig. 3. Kinetics of JNK1/2 phosphorylation in 3T3-L1 adipocytes treated with chemical inducers of IR: a) representative Western blots; b) time dependence of JNK1/2 phosphorylation at Thr183 and Tyr185.

Studies in 1990s and 2000s revealed that an excess of lipids in muscle cells causes activation of atypical isoforms of protein kinase C that interrupt signaling from the insulin receptor to the glucose transporter GLUT4 [39, 40]. However, a high lipid background in adipocytes suggests another mechanism for impairment of the insulin signaling, which emerged after identification of FFAs as activators of Toll-like 4 type receptors (TLR4) [41, 42]. These receptors are linked to activation of inflammatory signaling and inflammatory kinases IKK and JNK1/2 in cells. The role of these kinases in impairment of insulin signaling is now well documented [15]. The key role of JNK in serine phosphorylation of IRS and IR development in adipose tissue has been convincingly demonstrated in animal and cellular models of ER stress and inflammation [16, 18].

Our results are consistent with this mechanism, as we have observed that lipid overloading decreases the insulin-stimulated phosphorylation of IRS at Tyr612 (which is necessary for further insulin signaling to the PI3K cascade [5]), the insulin-stimulated phosphorylation of Akt and AS160 (Fig. 1), and glucose transport into adipocytes (Fig. 2). Treatment of cells with FFA or LPS increased the basal level of Ser473 phosphorylation in Akt (Fig. 1c), which may result from either direct phosphorylation by JNK1/2 [18] or their effects on activation of mTORC2 [15, 16]. Indeed, we have observed a prolonged activation of JNK1/2 under cell treatment with FFA (Fig. 3). However, we cannot rule out other mechanisms underlying the effects of FFAs, since the action of FFAs on adipocytes involves many complex processes [41, 42].

Mechanisms of the hypoxia-induced decrease in the insulin cascade activity. Obesity and IR development are associated with the hypertrophy of adipose tissue and hypoxia can induce IR in adipocytes [10]. The transcription factor HIF-1α is responsible for the cell response to hypoxia. Inhibition of von Hippel–Lindau proteins by Co^{2+} ions prevents proteasomal degradation of and stabilizes the HIF-1α [43, 44]. The transcription factor NF-κB is one of the target genes for HIF complex. Stabilization of HIF-1α facilitates NF-κB-dependent inflammatory mechanisms [45, 46]. Moreover, Co^{2+} ions stimulate production of reactive oxygen species and promote oxidative stress in the cells [47]. Therefore, hypoxia leads to activation of the inflammatory and stress-dependent kinases, including IKK and JNK1/2.

We found that Co^{2+} ions reduce the insulin-dependent phosphorylation of IRS, Akt (at Thr308), and AS160 (Fig. 1). As mentioned above, the increase in basal Akt phosphorylation at Ser473 (Fig. 1c) can be due to the activation of JNK1/2 [15, 16, 18]. Indeed, Co^{2+} caused a prolonged activation of JNK1/2 (Fig. 3), which indicates induction of inflammatory response. Despite decreased JNK1/2 activation by the end of the experiment, the insulin-stimulated glucose uptake remained significantly reduced (Fig. 2). Hence, experimental hypoxia induced IR development and affected glucose uptake in the adipocytes, probably due to the activation of long inflammatory response and involvement of JNK1/2.

Mechanisms of the ER stress-induced decrease in the insulin cascade activity. BrA affects the delivery of COPI-coated vesicles from the ER into the Golgi apparatus thus leading to protein overload and ER stress [48]. The mechanism of BrA action involves inflammatory transcription factor NF-κB [49].

BrA decreased the insulin-dependent phosphorylation of IRS, Akt (at Thr308), and AS160 (Fig. 1), with no effect only on Akt phosphorylation at Ser473 (Fig. 1c). It also significantly reduced stimulation of glucose uptake by insulin (Fig. 2). The kinetics of JNK1/2 activation were sustained, which is in agreement with the JNK1/2 role in serine phosphorylation of IRS and IR induction in adipocytes. Therefore, modeling the ER stress also leads to the inflammatory response and impairs glucose transport into adipocytes.

Mechanisms of the inflammation-induced decrease in the insulin cascade activity. A quite clear view has shaped of the relationship between obesity, inflammation, and IR development in the adipose tissue [19, 50]. The key feature of the IR development mechanism is a sustained (latent) character of inflammation (for details see [9, 12]).

The classic induction of inflammation with LPS led to the suppression of insulin signaling in adipocytes (Fig. 1) but did not cause a significant decrease in the insulin-stimulated glucose uptake (Fig. 2). LPS rapidly activated JNK1/2, but this effect disappeared already within 4 h, and the second, prolonged phase of JNK1/2 activation was virtually absent (Fig. 3). This is consistent with acute inflammatory response upon activation of the IKK/NF-κB signaling cascade through the TLR4 receptor on adipocytes. It is possible that no effect of LPS on the insulin-stimulated 2-DOG uptake (Fig. 2) maybe due to the absence of prolonged activation of JNK1/2 or different involvement of JNK1/2 isoforms. While JNK2 phosphorylation was affected by FFA, Co^{2+} and BrA, LPS mainly influenced phosphorylation of JNK1 (Fig. 3). The JNK1/2 isoforms can be differently involved in regulation of metabolism. Thus, the insulin cascade response to inflammation was fully absent in JNK1-deficient (but not in JNK2-deficient) mice [16]. However, this effect needed the presence of JNK2 and was affected by the JNK2 knockout [18]. Therefore, both JNK isoforms play important, but different, roles in the development of inflammation-induced IR [50].

As an acute inflammation inductor, LPS only transiently activated JNK1/2, and this seems to be insufficient to critically impair functions of AS160 and GLUT4. That LPS decreases AS160 phosphorylation at Ser318 (Fig. 1d) may not necessarily reflect changes in the AS160 activity. Although Ser318 is the major Akt phosphorylation site in AS160, AS160 has several other residues recognized by Akt [33, 34]. Moreover, the activity of AS160 is regulated by other kinases. In particular, AMP-dependent kinase (AMPK) regulates glucose transport in myocytes through phosphorylation of another set of residues in AS160 [51-53].

In this work, we have shown that modeling obesity-specific cellular pathologies such as dyslipidemia, hypoxia, and ER stress in 3T3-L1 adipocytes induces prolonged inflammatory response manifested by activation of stress-dependent kinases JNK1/2 and reduction of insulin activation of insulin cascade and glucose transport into the cells. By contrast, modeling of acute inflammation with LPS led to the transient activation of JNK1/2 and decreased activation of insulin signaling but did not affect glucose uptake by adipocytes. These results extend our understanding of the relationship between mechanisms of inflammation and IR and suggest that inflammatory process can be a promising target for new therapeutic strategies to correct insulin resistance and type 2 diabetes mellitus.

Funding

The work was supported by the Russian Foundation for Basic Research (project no. 14-35-00026) and National Intellectual Development Foundation for the Support of Scientific Project Activities of Students, Post-Graduates, and Young Scientists (project no. 17-34-80026 “mol_ev_a”).

Conflict of Interest

The authors declare the absence of pronounced and potential conflicts of interest associated with the publication of this paper.

Ethical Approval

This article does not include description of research carried out with the participation of people or animal subjects.

REFERENCES

1. *IDF Diabetes Atlas* (2017) 8th Edn. (http://diabetesatlas.org/resources/2017-atlas.html).
2. Dedov, I. I., Shestakova, M. V., Vikulova, O. K., Zheleznyakova, A. V., and Isakov, M. A. (2018) Diabetes mellitus in Russian Federation: distribution, morbidity, mortality, carbohydrate metabolism parameters and the structure of glucose-lowering therapy according to data of the Federal Register of diabetes mellitus, status 2017, *Sakharnyi Diabet*, **21**, 144-159; doi: 10.14341/DM9686.
3. Dedov, I. I., Shestakova, M. V., Peterkova, V. A., Vikulova, O. K., Zheleznyakova, A. V., Isakov, M. A., Laptev, D. N., Andrianova, E. A., and Shiryaeva, T. Y. (2017) Diabetes mellitus in children and adolescents according to data of the Federal Register of Russian Federation: dynamics of the main epidemiological characteristics during 2013-2016, *Sakharnyi Diabet*, **20**, 392-402; doi: 10.14341/DM9460.
4. Wilcox, G. (2005) Insulin and insulin resistance, *Clin. Biochem. Rev.*, **26**, 19-39.
5. Esposito, D. L., Li, Y., Cama, A., and Quon, M. J. (2001) Tyr612 and Tyr632 in human insulin receptor substrate-1 are important for full activation of insulin-stimulated phosphatidylinositol 3-kinase activity and translocation of GLUT4 in adipose cells, *Endocrinology*, **142**, 2833-2840; doi: 10.1210/endo.142.7.8283.
6. Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., Brown, M., Fitzgerald, K. J., and Sabatini, D. M. (2006) Ablation in mice of the mTORC components Raptor, Rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCα, but not S6K1, *Devel. Cell*, **11**, 859-871; doi: 10.1016/j.devcel.2006.10.007.
7. Tkachuk, V. A., and Vorotnikov, A. V. (2014) Molecular mechanisms of insulin resistance development, *Sakharnyi Diabet*, **17**, 29-41; doi: 10.14341/DM2014229-40.
8. Boura-Halfon, S., and Zick, Y. (2009) Phosphorylation of IRS proteins, insulin action, and insulin resistance, *Am. J. Physiol. Endocrinol. Metab.*, **296**, E581-E591; doi: 10.1152/ajpendo.90437.2008.
9. Stafeev, I. S., Menshikov, M. Y., Tsokolaeva, Z. I., Shestakova, M. V., and Parfyonova, Y. V. (2015) Molecular mechanisms of latent inflammation in metabolic syndrome. Possible role of sirtuins and peroxisome proliferator activated receptor type gamma, *Biochemistry (Moscow)*, **80**, 1217-1226; doi: 10.1134/S0006297915100028.
10. Trayhurn, P. (2013) Hypoxia and adipose tissue function and dysfunction in obesity, *Physiol. Rev.*, **93**, 1-21; doi: 10.1152/physrev.00017.2012.
11. Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., Tunchman, G., Gorgun, C., Glimcher, L. H., and Hotamisligil, G. S. (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes, *Science*, **306**, 457-461; doi: 10.1126/science.1103160.
12. Stafeev, I. S., Vorotnikov, A. V., Ratner, E. I., Menshikov, M. Y., and Parfyonova, Y. V. (2017) Latent inflammation and insulin resistance in adipose tissue, *Int. J. Endocrinol.*, **2017**, 1-12; doi: 10.1155/2017/5076732.
13. Zick, Y. (2004) Uncoupling insulin signalling by serine/threonine phosphorylation: a molecular basis for insulin resistance, *Biochem. Soc. Trans.*, **32**, 812-816; doi: 10.1042/BST0320812.
14. Morino, K., Petersen, K. F., and Shulman, G. I. (2006) Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction, *Diabetes*, **55**, S9-S15; doi: 10.2337/db06-S002.
15. Taniguchi, C. M., Emanuelli, B., and Kahn, C. R. (2006) Critical nodes in signalling pathways: insights into insulin action, *Nat. Rev. Mol. Cell. Biol.*, **7**, 85-96; doi: 10.1038/nrm1837.
16. Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M., and Hotamisligil, G. S. (2002) A central role for JNK in obesity and insulin resistance, *Nature*, **420**, 333-336; doi: 10.1038/nature01137.
17. Arkan, M. C., Hevener, A. L., Greten, F. R., Maeda, S., Li, Z. W., Long, J. M., Wynshaw-Boris, A., Poli, G., Olefsky, J., and Karin, M. (2005) IKK-beta links inflammation to obesity-induced insulin resistance, *Nat. Med.*, **11**, 191-198; doi: 10.1038/nm1185.
18. Tuncman, G., Hirosumi, J., Solinas, G., Chang, L., Karin, M., and Hotamisligil, G. S. (2006) Functional *in vivo* interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance, *Proc. Natl. Acad. Sci. USA*, **103**, 10741-10746; doi: 10.1073/pnas.0603509103.
19. Saltiel, A. R., and Olefsky, J. M. (2017) Inflammatory mechanisms linking obesity and metabolic disease, *J. Clin. Invest.*, **127**, 1-4; doi: 10.1172/JCI92035.
20. Donath, M. Y., and Shoelson, S. E. (2011) Type 2 diabetes as an inflammatory disease, *Nat. Rev. Immunol.*, **11**, 98-107; doi: 10.1038/nri2925.
21. Saltiel, A. R. (2016) Insulin signaling in the control of glucose and lipid homeostasis, *Handb. Exp. Pharmacol.*, **233**, 51-71; doi: 10.1007/164_2015_14.
22. Lackey, D. E., and Olefsky, J. M. (2016) Regulation of metabolism by the innate immune system, *Nat. Rev. Endocrinol.*, **12**, 15-28; doi: 10.1038/nrendo.2015.189.
23. Shimobayashi, M., Albert, V., Woelnerhanssen, B., Frei, I. C., Weissenberger, D., Meyer-Gerspach, A. C., Clement, N., Moes, S., Colombi, M., Meier, J. A., Swierczynska, M. M., Jeno, P., Beglinger, C., Peterli, R., and Hall, M. N. (2018) Insulin resistance causes inflammation in adipose tissue, *J. Clin. Invest.*, **128**, 1538-1550; doi: 10.1172/JCI96139.
24. Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z. W., Karin, M., and Shoelson, S. E. (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta, *Science*, **293**, 1673-1677; doi: 10.1126/science.1061620.
25. Donath, M. Y. (2014) Targeting inflammation in the treatment of type 2 diabetes: time to start, *Nat. Rev. Drug Disc.*, **13**, 465-476; doi: 10.1038/nrd4275.
26. Stafeev, I. S., Michurina, S. S., Podkuychenko, N. V., Vorotnikov, A. V., Menshikov, M. Y., and Parfyonova, Y. V. (2018) Interleukin-4 restores insulin sensitivity in lipid-induced insulin resistant adipocytes, *Biochemistry (Moscow)*, **83**, 498-506; doi: 10.1134/S0006297918050036.
27. Zebisch, K., Voight, V., Wabitsch, M., and Brandsch, M. (2012) Protocol for effective differentiation of 3T3L1 cells to adipocytes, *Analyt. Biochem.*, **425**, 88-90; doi: 10.1016/j.ab.2012.03.005.
28. She, M., Hou, H., Wang, Z., Zhang, C., Laudon, M., and Yin, W. (2014) Melatonin rescues 3T3-L1 adipocytes from FFA-induced insulin resistance by inhibiting phosphoryla-

tion of IRS-1 on Ser307, *Biochimie*, **103**, 126-130; doi: 10.1016/j.biochi.2014.05.001.
29. Svedberg, J., Bjorntorp, P., Smith, U., and Lonnroth, P. (1990) Free-fatty acid inhibition of insulin binding, degradation, and action in isolated rat hepatocytes, *Diabetes*, **39**, 570-574.
30. Citterio, C., Vichi, A., Pacheco-Rodriguez, G., Aponte, A. M., Moss, J., and Vaughan, M. (2008) Unfolded protein response and cell death after depletion of brefeldin A-inhibited guanine nucleotide-exchange protein GBF1, *Proc. Natl. Acad. Sci. USA*, **105**, 2877-2882; doi: 10.1073/pnas.0712224105.
31. Glassford, A. J., Yue, P., Sheikh, A. Y., Chun, H. J., Zarafshar, S., Chan, D. A., Reaven, G. M., Quertermous, T., and Tsao, P. S. (2007) HIF-1 regulates hypoxia- and insulin-induced expression of apelin in adipocytes, *Am. J. Physiol. Endocrinol. Metab.*, **293**, E1590-1596; doi: 10.1152/ajpendo.00490.2007.
32. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**, 680-685.
33. Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., Garner, C. C., and Lienhard, G. E. (2003) Insulin-stimulated phosphorylation of Rab GTPase-activating protein regulates GLUT4 translocation, *J. Biol. Chem.*, **278**, 14599-14602; doi: 10.1074/jbc.C300063200.
34. Kane, S., Sano, H., Liu, S. C. H., Asara, J. M., Lane, W. S., Garner, C. C., and Lienhard, G. E. (2002) A method to identify serine kinases substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain, *J. Biol. Chem.*, **277**, 22115-22118; doi: 10.1074/jbc.C200198200.
35. Randle, P. J., Garland, P. B., Hales, C. N., and Newsholme, E. A. (1963) The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus, *Lancet*, **1**, 785-789.
36. Randle, P. J. (1998) Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years, *Diabetes Metab. Rev.*, **14**, 263-283.
37. Cole, T. G., Patsch, W., Kuisk, I., Gonen, B., and Schonfeld, G. (1983) Increases in dietary cholesterol and fat raise levels of apoprotein E-containing lipoproteins in the plasma of man, *J. Clin. Endocrinol. Metab.*, **56**, 1108-1115; doi: 10.1210/jcem-56-6-1108.
38. Golay, A., and Bobbioni, E. (1997) The role of dietary fat in obesity, *Int. J. Obes. Relat. Metab. Disord.*, **21**, Suppl. 3, S2-S11.
39. Samuel, V. T., Petersen, K. F., and Shulman, G. I. (2010) Lipid-induced insulin resistance: unravelling the mechanism, *Lancet*, **375**, 2267-2277; doi: 10.1016/S0140-6736(10)60408-4.
40. Shulman, G. I. (2014) Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease, *N. Engl. J. Med.*, **371**, 1131-1141; doi: 10.1056/NEJMra1011035.
41. Oeckinghaus, A., Hayden, M. S., and Ghosh, S. (2011) Crosstalk in NF-kappaB signaling pathways, *Nat. Immunol.*, **12**, 695-708; doi: 10.1038/ni.2065.
42. Lancaster, G. I., Langley, K. G., Berglund, N. A., Kammoun, H. L., Reibe, S., Estevez, E., Weir, J., Mellett, N. A., Pernes, G., Conway, J. R. W., Lee, M. K. S., Timpson, P., Murphy, A. J., Masters, S. L., Gerondakis, S., Bartonicek, N., Kaczorowski, D. C., Dinger, M. E., Meikle, P. J., Bond, P. J., and Febbraio, M. A. (2018) Evidence that TLR4 is not a receptor for saturated fatty acids but mediates lipid-induced inflammation by reprogramming macrophage metabolism, *Cell Metab.*, **27**, 1096-1110; doi: 10.1016/j.cmet.2018.03.014.
43. Weidemann, A., and Johnson, R. S. (2008) Biology of HIF-1α, *Cell. Death Differ.*, **15**, 621-627; doi: 10.1038/cdd.2008.12.
44. Greer, S. N., Metcalf, J. L., Wang, Y., and Ohh, M. (2012) The updated biology of hypoxia inducible factor, *EMBO J.*, **31**, 2448-2460; doi: 10.1038/emboj.2012.125.
45. Melillo, G. (2011) Hypoxia: jump-starting inflammation, *Blood*, **117**, 2561-2562; doi: 10.1182/blood-2010-12-324913.
46. Eltzschig, H. K., and Carmeliet, P. (2011) Hypoxia and inflammation, *N. Engl. J. Med.*, **364**, 656-665; doi: 10.1056/NEJMra0910283.
47. Kamiya, T., Hara, H., Inagaki, N., and Adachi, T. (2010) The effect of hypoxia mimetic cobalt chloride on the expression of EC-SOD in 3T3-L1 adipocytes, *Redox. Rep.*, **15**, 131-137; doi: 10.1179/174329210X12650506623483.
48. Anadu, N. O., Davisson, V. J., and Cushman, M. (2006) Synthesis and anticancer activity of brefeldin A ester derivatives, *J. Med. Chem.*, **49**, 3897-3905; doi: 10.1021/jm0602817.
49. Pahl, H. L., and Baeuerle, P. A. (1995) A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF-kappa B, *EMBO J.*, **14**, 2580-2588.
50. Hotamisligil, G. S. (2006) Inflammation and metabolic disorders, *Nature*, **444**, 860-867; doi: 10.1038/nature05485.
51. Treebak, J. T., Glund, S., Deshmukh, A., Klein, D. K., Long, Y. C., Jensen, T. E., Jorgensen, S. B., Viollet, B., Andersson, L., Neumann, D., Wallimann, T., Richter, E. A., Chibalin, A. V., Zierath, J. R., and Wojtaszewski, J. F. (2006) AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits, *Diabetes*, **55**, 2051-2058; doi: 10.2337/db06-0175.
52. Kramer, H. F., Witczak, C. A., Fujii, N., Jessen, N., Taylor, E. B., Arnolds, D. E., Sakamoto, K., Hirshman, M. F., and Goodyear, L. J. (2006) Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle, *Diabetes*, **55**, 2067-2076; doi: 10.2337/db06-0150.
53. Treebak, J. T., Birk, J. B., Rose, A. J., Kiens, B., Richter, E. A., and Wojtaszewski, J. F. (2007) AS160 phosphorylation is associated with activation of alpha2beta2gamma1- but not alpha2beta2gamma3-AMPK trimeric complex in skeletal muscle during exercise in humans, *Am. J. Physiol. Endocrinol. Metab.*, **292**, E715-E722; doi: 10.1152/ajpendo.00380.2006.