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Dimethylaminopurine inhibits metabolic effects of insulin in primary adipocytes

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Abstract

Dimethylaminopurine (DMAP) has previously been used as an inhibitor of phosphorylation in studies of meiotic events, and more recently to investigate TNFα signaling, because of its potential to inhibit activation of c-jun N-terminal kinase (JNK). Here we have addressed the effects of DMAP on metabolic insulin responses in adipocytes and on intracellular insulin signaling molecules.

At 100 μmol/L, DMAP completely inhibited the ability of insulin to counteract lipolysis in isolated adipocytes. Insulin-induced lipogenesis and glucose uptake was inhibited to a lesser degree in a concentration-dependent manner starting at 10 μmol/L DMAP. Insulin-induced tyrosine phosphorylation of the insulin receptor was not affected by DMAP. Insulin-induced activation of protein kinase B, a known mediator of insulin action, was not inhibited by 100 μmol/L, but to a low extent by 1 mmol/L DMAP in intact cells. This inhibition was not sufficient to affect activation of the downstream protein kinase B substrate phosphodiesterase 3B.

The inhibition of activation of JNK as a possible mechanism whereby DMAP affects insulin-induced antilipolysis, lipogenesis, and glucose uptake, was investigated using the JNK inhibitor SP600125. At 100 μmol/L, SP600125 completely reversed the antilipolytic effect of insulin, as well as partially inhibited insulin-induced lipogenesis and glucose-uptake, indicating that JNK may be involved in mediating these actions of insulin. Inhibition of JNK by DMAP may therefore partly explain the negative impact of DMAP on insulin action in adipocytes.

Keywords: DMAP; PKB; JNK; Antilipolysis; Insulin; Adipocyte

1. Introduction

Altered metabolism and insulin resistance in adipose tissue is of importance for the development of type 2 diabetes and obesity. To understand fully and to prevent these diseases, studies of adipocyte function and molecular mechanisms of insulin action are of great importance.

The storage of triglycerides in adipose tissue is promoted by insulin, mainly because of its ability to counteract catecholamine-induced lipolysis. Phosphodiesterase 3B (PDE 3B) is a key enzyme in the antilipolytic signaling pathway of insulin [1,2]. PDE 3B is phosphorylated and activated in a phosphoinositide-3 kinase (PI3-K)–dependent manner in response to insulin [3,4], leading to lowered cAMP levels in the cell. This in turn results in decreased activity of protein kinase A and consequently hormone sensitive lipase (HSL), the rate-limiting enzyme in triglyceride breakdown [5]. The kinase downstream of PI3-K responsible for the phosphorylation and activation of PDE 3B is protein kinase B (PKB) [6-8]. PKB in rat adipocytes is activated in response to insulin by phosphorylation mainly on Ser-474 (PKBβ) [9].

Other ways in which insulin promotes lipid storage in fat cells is by increasing glucose uptake and lipid formation, the latter both by stimulating fatty acid re-esterification and *de novo* lipid synthesis. The mechanism whereby insulin induces glucose uptake has been extensively studied but is still not fully understood. PI3-K is thought to be an important component of the pathway [10], but PI3-K–independent insulin-induced glucose uptake has also been demonstrated [11]. In several studies, insulin-induced glucose uptake has been shown to involve PKB [12–16], although the relative importance of PKB for this pathway is debated [17]. PKB has also been demonstrated to be important for the lipogenic effect of insulin [12].

In an initial screening performed to test compounds for

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their ability to influence adipose tissue metabolism, dimethylaminopurine (DMAP) was identified as an agent of interest. DMAP has previously been used as a general inhibitor of phosphorylation to investigate various meiotic events in oocytes [18–20]. More recently DMAP has been shown to be a blocker of activation of the mitogen-activated protein kinase (MAPK) member c-jun N-terminal kinase (JNK), also known as stress-activated protein kinase. JNK is activated in response to cytokines and cellular stress, and regulates apoptosis as well as survival signals through its action on the activator protein-1 (AP-1) transcription complex [21]. Recently JNK has also been shown to be activated by insulin [22–25], and a role for JNK as a mediator of insulin effects, more specifically activation of glycogen synthase, has been demonstrated [26]. Recent studies also suggest that JNK functions as a negative regulator of the insulin signaling pathway, most likely through serine phosphorylation of insulin receptor substrate [25,27].

DMAP has been used as an inhibitor of JNK activation in studies of TNFα signaling in endothelial cells [28], Sertoli cells [29], and fat cells [30]. However, possible actions of this inhibitor on other signaling pathways have not been investigated, and no direct cellular targets of DMAP have so far been identified.

In this study we have examined the effect of DMAP on metabolic effects and signaling pathways in rat adipocytes. To investigate the mechanism by which DMAP exerts these effects, we have also studied the impact of DMAP on known mediators of insulin signaling such as the insulin receptor (IR), PKB, and PDE 3B. In addition, we have used the selective JNK inhibitor SP600125 to investigate whether DMAP could mediate its effects via an inhibition of JNK.

2. Methods and materials

2.1. Drugs

DMAP and isoprenalin was obtained from Sigma (St. Louis, MO). Insulin was obtained from Novo Nordisk (Copenhagen, Denmark) and SP600125 from Biomol (Plymouth Meeting, MA). DMAP and SP600125 were dissolved in DMSO.

2.2. Preparation, stimulation, and homogenization of adipocytes

Adipocytes prepared from epididymal adipose tissue of 36–38 days old male Sprague-Dawley rats (B&K Universal, Stockholm, Sweden), [31,32] were suspended in Krebs-Ringer medium pH 7.4, 25 mmol/L HEPES pH 7.4, 200 nmol/L adenosine, 2 mmol/L glucose, and 1% BSA, and were incubated (typically 2 mL of a 10% suspension) at 37°C and stimulated as indicated in figures 4, 5, 6, 7. At indicated times, cells were washed and subsequently homogenized in 1 mL buffer consisting of 50 mmol/L Tris pH 7.5, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 270 mmol/L sucrose, 0,1% β-mercaptoethanol, 1 μmol/L microcystin-LR (Alexis Biochemicals, Montreal, PQ, Canada), 1 μg/mL pepstatin and 10 μg/mL each of leupeptin and antipain (buffer A, JNK and insulin receptor (IR) measurements) or 50 mmol/L TES pH 7.4, 2 mmol/L EGTA, 1 mmol/L EDTA, 250 mmol/L sucrose, 40 mmol/L phenylphosphate, 5 mmol/L NaF, 1 mmol/L DTE, 1 mmol/L PMSF, 50 μmol/L sodium orthovanadate, 500 nmol/L okadaic acid (LC Laboratories, Switzerland), 1 μg/mL pepstatin, and 10 μg/mL each of leupeptin and antipain (buffer B, PKB, and PDE 3B measurements). A crude membrane fraction was then prepared by centrifugation at 16,600 $\times$ g for 20 minutes (JNK measurements) or 33,000 $\times$ g for 45 minutes (PKB and PDE 3B measurements), at 4°C. The fat cake was removed, the infranatant (referred to as cytosol fraction) withdrawn and the pellet (referred to as membrane fraction) was resuspended and rehomogenized in 500 μL of a buffer containing 50 mmol/L TES pH 7.4, 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L EGTA, 100 nmol/L okadaic acid, 1 μg/mL pepstatin, and 10 μg/mL each of leupeptin and antipain. Alternatively (for IR measurements), homogenates free from fat were supplemented with 1% Triton X-100 (final concentration) and solubilized for 1 hour on ice. Insoluble material was removed by centrifugation at 13,000 rpm for 10 minutes at 4°C.

2.3. Measurements of lipolysis, lipogenesis, and glucose uptake in adipocytes

To measure lipolysis adipocytes (0.4–1 mL of 5% (v/v) suspension) were incubated in Krebs-Ringer medium pH 7.4, 25 mmol/L HEPES pH 7.4, 200 nmol/L adenosine, 2 mmol/L glucose, and 1% bovine serum albumin, at 37°C with indicated additions (with shaking, 150 cycles/min). After 30 minutes the cells were placed on ice for 20 minutes, and 200 μL of the cell medium was removed for enzymatic determination of the glycerol content, as described previously [33]. Lipogenesis was assayed in 1 mL of a 2% (v/v) suspension of adipocytes in Krebs-Ringer medium pH 7.4, 25 mmol/L HEPES pH 7.4, 200 nmol/L adenosine, 0.55 mmol/L glucose, and 3.5% BSA, as the incorporation of ^{3}H-glucose into adipocyte triglycerides [34] under a 30-minute incubation. 2-Deoxy ^{3}H-glucose uptake was measured in 100 μL of a 30% (v/v) suspension of adipocytes in 120 mmol/L NaCl, 4 mmol/L KH_2PO_4, 1 mmol/L $MgSO_4$, 0.75 mmol/L $CaCl_2$, 10 mmol/L $NaHCO_3$, 30 mmol/L HEPES pH 7.4, and 1% BSA, as described elsewhere [35].

2.4. Immunoprecipitation and Western blot analysis

Immunoprecipitation of the IR from adipocyte homogenates (200 μL packed cell volume [PCV]) was carried out at

4°C overnight using 3 μg of an anti IR antibody (Upstate Biotechnology Inc., Lake Placid, NY). Immunocomplexes were collected by incubation with 20 μL Protein G Sepharose for 30–60 minutes, washed three times with PBS containing 0.1% (v/v) N-laurylsarcosine, and boiled in Laemmli sample buffer. Immunoprecipitates (IR) and adipocyte cytosol fractions (PKB) were subjected to SDS-PAGE (7 and 9% acrylamide respectively) followed by electrotransfer of proteins onto nitrocellulose membranes. Membranes were blocked for 1 hour with 0.5% gelatin (IR) or 10% dry milk (PKB) in 20 mmol/L Tris pH 7.6, 137 mmol/L NaCl and 0.1% (w/v) Tween-20 for 30–60 minutes, and were incubated for 16 hours with the primary antibodies anti phosphotyrosine (PY99, Santa Cruz Biotechnology, Santa Cruz, CA) or anti phospho-Akt (Ser473 and Thr308) at a 1:2000 dilution (Cell Signaling Technology, Beverly, MA). Immunoblot analysis was performed using the Super Signal reagent (Pierce, Rockford, IL), and the chemiluminescent light was captured using a cooled CCD camera (LAS 1000 Plus, Fuji, Tokyo, Japan). The relative intensities of the bands were determined using the Image Gauge Software (Fuji, Tokyo, Japan).

2.5. Isolation of ^{32}P-labeled PKBβ from adipocytes

Isolated primary rat adipocytes were suspended (10 mL of 12% suspension) in low-phosphate Krebs-Ringer medium containing 25 mmol/L HEPES pH 7.4, 200 nmol/L adenosine, 2 mmol/L glucose, 3.5% BSA, and 300 μmol/L $KH_2PO_4$and incubated at 37°C with 0.5 mCi ^{32}P/mL (Amersham Pharmacia Biotech, Little Chalfont, UK) for 1 hour. Cells were stimulated with insulin in the absence or presence of DMAP as indicated in Figure 5, and washed and homogenized in buffer B. Cytosol fractions were prepared by centrifugation for 45 minutes at 33,000 × *g* and subjected to immunoprecipitation using 2 μg/240 μL PCV of an anti PKBβ antibody (Upstate Biotechnology, Lake Placid, NY). Subsequently, immunoprecipitates were washed and subjected to SDS-PAGE and electrotransfer to PVDF membrane followed by detection of ^{32}P by digital imaging (Fujix BAS 2000, Fuji, Tokyo, Japan). The relative intensities of the bands were determined using Image Gauge Software (Fuji, Tokyo, Japan).

2.6. Assay of PKB

PKB-containing cytosol fractions (10 μL) from insulin stimulated adipocytes were incubated for 20 minutes at 30°C with 5 μL of a mixture containing 17 mmol/L TES pH 7.5, 42 mmol/L $MgSO_4$, 4.2 mmol/L dithioerythriol, 207 mmol/L sucrose, 170 μmol/L [γ-^{32}P]ATP (specific activity 30,000–40,000 cpm/pmol ATP), 6.6 μmol/L cAMP-dependent protein kinase inhibitor, 13 μg of the peptide substrate RPRAATF and DMAP at indicated concentrations. Incubations were terminated by the addition of 10 μL of 1% BSA, 1 mmol/L ATP, pH 3.0, and 5 μL of 30% trichloroacetic acid. After 15 minutes on ice, samples were centrifuged and 15 μL of the supernatants was applied to phosphocellulose paper (Whatman P81) that was washed five times with 75 mmol/L phosphoric acid and once with acetone. The amount of ^{32}P incorporated into the peptide substrate was determined by scintillation counting.

2.7. Assay of JNK

Adipocyte cytosol fractions (300–500 μL) were incubated for 2 hours with 3 μg of an anti JNK1 antibody (Upstate Biotechnology, Lake Placid, NY). The last 30 minutes protein G sepharose was included to collect the immunocomplexes, which were subsequently washed twice with a buffer containing 50 mmol/L Tris pH 7.5, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton-X 100, 1 mmol/L sodium orthovanadate, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 270 mmol/L sucrose, and 0.5 M NaCl, and twice with a buffer containing 50 mmol/L Tris pH 7.5, 0.1 mmol/L EDTA, and 0.1% β-mercaptoethanol. Immunoprecipitates were then incubated for 20 minutes at 30 °C in a total volume of 50 μL consisting of 50 mmol/L Tris pH 7.5, 0.1% β-mercaptoethanol, 0.1 mmol/L EDTA, 2.5 μmol/L cAMP-dependent protein kinase inhibitor, 500 nmol/L microcystin-LR (Alexis Biochemicals, Montreal, PQ, Canada), 10 mmol/L $MgCl_2$, 100 μmol/L [γ-^{32}P]ATP (specific activity 250–500 cpm/pmol ATP), and 3 μmol/L of the peptide substrate ATF2 (Upstate Biotechnology, Lake Placid, NY). The reactions were stopped by applying 40 μL of the reactions onto phosphocellulose paper (Whatman P81) that was washed five times with 75 mmol/L phosphoric acid and once with acetone. The amount of ^{32}P incorporated into the peptide substrate was determined by scintillation counting.

2.8. Statistical methods

The statistical significance of differences was analyzed with the Student's *t* test (paired). $P < 0.05$ was used as the cut-off point for significance. Results are presented as means ± SD.

3. Results

An important metabolic effect of insulin is to promote the storage of triglycerides by counteracting adipose tissue lipolysis induced by cAMP-increasing agents. In a search for compounds with effects on insulin-mediated regulation of lipolysis, we identified DMAP as a novel modulator of insulin action in adipocytes.

3.1. DMAP inhibits insulin-induced antilipolysis in adipocytes

To investigate the effect of DMAP on insulin-induced antilipolysis, isolated primary adipocytes were stimulated

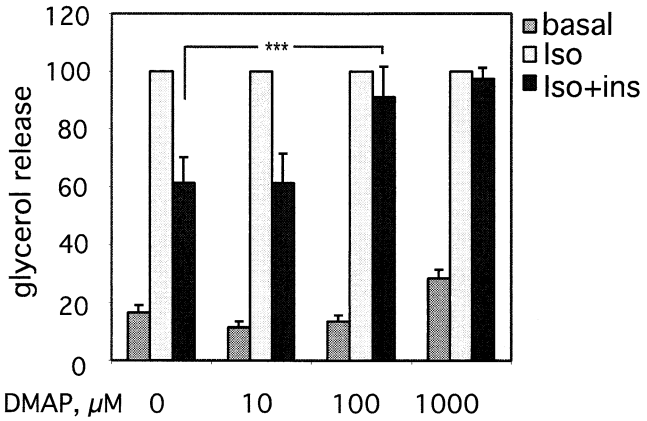

Fig. 1. Inhibition of the antilipolytic effect of insulin by DMAP in primary rat adipocytes. Primary rat adipocytes were preincubated for 30 minutes with or without different concentrations of DMAP, and then stimulated with or without 100 nmol/L isoprenalin (iso) and with or without 1.7 nmol/L insulin (ins) for 30 minutes. Lipolysis was then measured as accumulated glycerol release. Because of the variation in absolute values, results are presented as percentage of the maximal value within each group of DMAP concentration, and are mean values ± SD from four independent experiments. ***$P < 0.001$. The absolute values for isoprenalin-induced lipolysis (taken as 100%) are: 0 DMAP; 352–469 nmol glycerol/30 min/50 μL PCV, 0.01 mmol/L DMAP; 348–452 nmol glycerol/30 min/50 μL PCV, 0.1 mmol/L DMAP; 338–431 nmol glycerol/30 min/50 μL PCV, 1 mmol/L DMAP; 348–467 nmol glycerol/30 min/50 μL PCV.

with the lipolytic agent isoprenalin, a β-adrenergic agonist, in the absence or presence of insulin and different doses of DMAP. As shown in Fig. 1, stimulation of adipocytes with isoprenalin resulted in increased lipolysis, measured as an increased glycerol release. In the absence of DMAP, insulin was able to counteract this effect. However, in the presence of increasing concentrations of DMAP, the antilipolytic action of insulin was inhibited in a concentration-dependent fashion. The full effect of DMAP on antilipolysis was obtained at 100 μmol/L, whereas at 10 μmol/L DMAP no effect was seen. Furthermore, at the maximal DMAP concentration 1 mmol/L, basal lipolysis was significantly increased.

3.2. DMAP inhibits insulin-induced lipogenesis in adipocytes

Insulin promotes lipid storage by stimulating triglyceride formation in adipose tissue. The effect of DMAP on this lipogenic action of insulin was examined. Primary rat adipocytes were stimulated with insulin in the absence and presence of DMAP, and lipogenesis was measured as increased incorporation of exogenously added ^{3}H-glucose into adipocyte triglycerides. As shown in Fig. 2, DMAP counteracted insulin-induced lipogenesis in a concentration-dependent manner. Half-maximal effect was seen using 300 μmol/L, and a significant 10% decrease was obtained already at 10 μmol/L DMAP. Basal lipogenesis was significantly decreased at 700 μmol/L DMAP and further inhibited at 1 mmol/L DMAP.

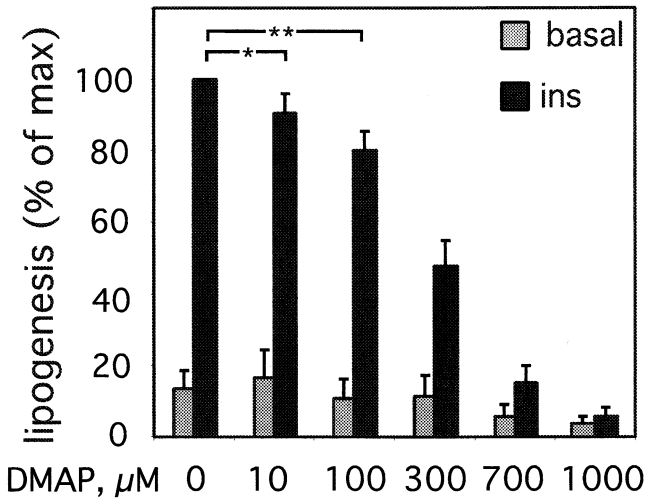

Fig. 2. Inhibition of insulin-induced lipogenesis in primary rat adipocytes. Primary rat adipocytes were preincubated for 30 minutes with or without DMAP at the concentrations indicated, and stimulated with or without 100 pmol/L insulin (ins) for 30 minutes. Lipogenesis was measured as the incorporation of ^{3}H-glucose into adipocyte neutral lipids. Results are presented as percent of the maximal value, and are means ± SD from four independent experiments. *$P < 0.05$, **$P < 0.01$.

3.3. DMAP inhibits insulin-induced glucose uptake in adipocytes

Another mechanism by which insulin increases the lipid stores is by stimulating uptake of glucose into adipocytes. The effect of DMAP on insulin-induced glucose uptake was measured as the uptake of 2-deoxy ^{3}H-glucose, a nonmetabolizable form of glucose, into primary adipocytes incubated with insulin in the absence or presence of increasing concentrations of DMAP. As shown in Fig. 3, the 2.5-fold

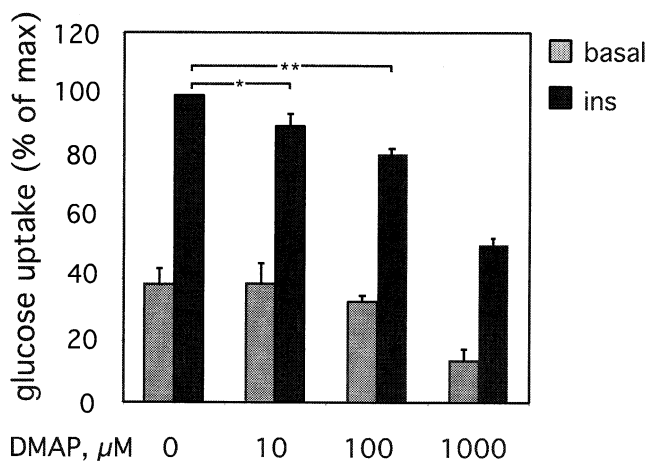

Fig. 3. Inhibition of insulin-induced glucose uptake in primary rat adipocytes. Primary rat adipocytes were preincubated for 30 minutes with or without different concentrations of DMAP, and stimulated with or without 1 nmol/L insulin (ins) for 30 minutes. Uptake of 2-deoxy ^{3}H-glucose from the medium was measured by scintillation counting of the cells. Results are presented as percentage of the maximal value, and are means ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$.

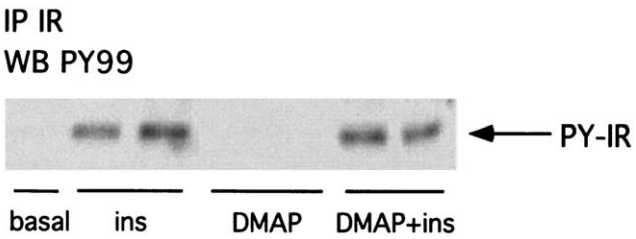

Fig. 4. DMAP does not influence insulin-induced tyrosine phosphorylation of the insulin receptor. Primary rat adipocytes were preincubated for 30 minutes with or without 1 mmol/L of DMAP, and stimulated with or without 100 nmol/L insulin (ins) for 10 minutes. The insulin receptor (IR) was immunoprecipitated (IP) from the homogenates, and tyrosine phosphorylation was analyzed by Western blot (WB) using phosphotyrosine (PY) antibodies. Results presented are representative of three independent experiments.

increase in glucose uptake obtained after insulin stimulation was inhibited in a concentration-dependent way by DMAP, with a significant 11% decrease at 10 μmol/L. Using the maximal DMAP concentration, 1 mmol/L, total glucose uptake was further inhibited, but the fold increase in response to insulin was maintained as compared to that in nontreated cells, because of the significant negative effects that this concentration of DMAP had on basal glucose uptake.

3.4. Effects of DMAP on selected known insulin signaling components

To explain the effects of DMAP on insulin action in adipocytes, we investigated the possible impact of DMAP on known mediators of insulin action, in particular the IR, PKB, and PDE 3B. To study the effect of DMAP on tyrosine phosphorylation of the IR, isolated adipocytes were stimulated with insulin in the absence or presence of 1 mmol/L DMAP. Tyrosine phosphorylation of the IR was then analyzed using phosphotyrosine antibodies. As seen in Fig. 4, insulin induced tyrosine phosphorylation of the receptor. Pretreatment of the cells with 1 mmol/L DMAP did not influence this phosphorylation, as shown in Fig. 4 and by means of quantification of the bands (data not shown). Thus, the effect of DMAP on insulin signaling was not at the level of the IR, and therefore we continued to investigate the impact of DMAP on the downstream effectors PKB and PDE 3B. Isolated adipocytes were stimulated with insulin in the absence or presence of increasing concentrations of DMAP, and after homogenization and centrifugation, PKB and PDE 3B activities were measured in cytosol and membrane fractions respectively. Figure 5a shows that pretreatment of adipocytes with 1 mmol/L DMAP causes a 30% inhibition of the insulin-induced PKB activity, whereas 10 and 100 μmol/L DMAP does not influence PKB activity. The inhibition at 1 mmol/L DMAP could either be due to an effect of DMAP on upstream signaling or to a direct inhibitory action of DMAP on PKB, with a binding strong enough to be maintained throughout the homogenization, centrifugation, and subsequent kinase assay. To investigate the nature and cause of the inhibition, PKB phosphorylation (Ser 473/474, Thr 308/309 and total) was monitored using phosphospecific antibodies as well as *in vivo* ^{32}P-labeling. As seen in Fig. 5b, there was a tendency toward a decrease in Ser 473 phosphorylation after DMAP pretreatment, but no inhibition of Thr 308 phosphorylation. As shown in Fig. 5c, and as judged by quantification of the signals from three experiments, there was a nonsignificant decrease (12%, $P = 0.37$) in total phosphorylation. The results in Figs. 5b and 5c suggest that DMAP to a limited extent may influence signaling components upstream of PKB, affecting PKB phosphorylation and activation. However, since this could only partially explain the 30% inhibition of PKB activity at 1 mmol/L DMAP, we also studied the direct effect of DMAP on PKB by incubating adipocyte PKB, in the absence or presence of increasing concentrations of DMAP *in vitro*. As seen in Fig. 5d, at 100μmol/L DMAP caused a 25% inhibition of PKB activity and at the maximal concentration 1 mmol/L, there was a 70% decrease in PKB activity. Given the high concentrations needed (IC50 $>$300 μmol/L), DMAP must be considered as a low potency inhibitor of PKB. This is in agreement with the high concentrations required to inhibit PKB in cells.

To monitor the consequences of PKB inhibition in intact cells, we also investigated the effect of DMAP treatment on the insulin-induced activation of PDE 3B, a downstream substrate for PKB. As shown in Fig. 6, pretreatment of adipocytes with 1 mmol/L DMAP did not influence insulin-induced activation of PDE 3B. The results in Fig. 5 and Fig. 6 together suggest that inhibition of PKB is not likely to be the mechanism whereby DMAP exerts its inhibitory effects on insulin action.

3.5. Possible involvement of JNK in mediating the effects of DMAP in adipocytes

DMAP has previously been described as an inhibitor of TNFα-induced activation of the MAPK member JNK [28,29]. Furthermore, JNK has also been shown to be activated by insulin [22–24]. To study the effect of DMAP on insulin-induced activation of JNK, primary adipocytes were insulin-stimulated with or without DMAP pretreatment, and an *in vitro* JNK1 immunoprecipitation kinase assay using ATF2 as a substrate, was performed. As seen in Fig. 7, stimulation of rat adipocytes with 10 nmol/L of insulin for 1 minute indeed resulted in a significant 2-fold increase in JNK kinase activity. This activation was transient, since no activation could be observed after a 10-minute insulin stimulation (data not shown). Pretreatment with DMAP caused a reversal of this activation. This result suggests that the mechanism whereby DMAP affects insulin-induced antilipolysis, lipogenesis and glucose uptake (Figs. 1–3) could involve inhibition of JNK. This hypothesis was tested by measuring insulin-induced antilipolysis, lipogenesis, and

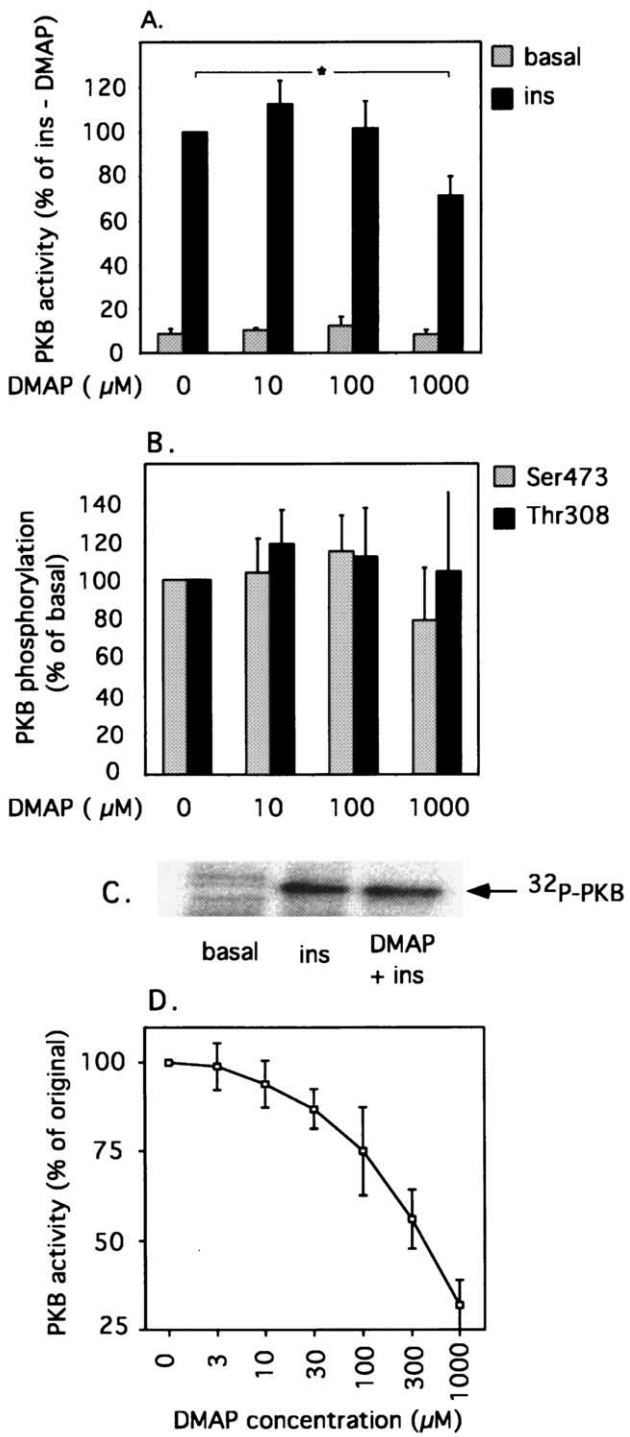

Fig. 5. Inhibition of insulin-induced PKB activity by DMAP. Primary rat adipocytes were preincubated for 30 minutes with or without different concentrations of DMAP, and stimulated with or without 1 nmol/L insulin (ins) for 10 minutes. Cytosol fractions were prepared and PKB activity (a) and Ser 473 and Thr 308 phosphorylation (b) was measured using a peptide assay and western blot analysis respectively. To monitor total PKB phosphorylation (c), cells were *in vivo* ^{32}P-labeled, stimulated as described above, and subsequently PKB (β) was immunoisolated, subjected to SDS-PAGE, and analyzed using phosphoimaging. Direct action of DMAP on PKB kinase activity (d) was measured by incubation of activated PKB *in vitro* in the presence of increasing concentrations of DMAP. In (a), (b), and (d), results are presented as percentage of the value obtained in the absence of inhibitor and are means ± SD of four independent experiments. In (b), the bands were detected using a cooled CCD camera and the relative intensities were determined using the Image Gauge Software (Fuji). (c) Representation of three independent experiments. The absolute value for insulin-induced PKB activity (taken as 100%) in (a) is 0.95–2.19 pmol ATP/min/2.5 μL PCV, and in (e) 0.20–0.71 pmol ATP/min/2.5 μL PCV. *$P < 0.05$

glucose uptake in the presence of increasing concentrations of the newly described JNK inhibitor SP600125 [36]. As shown in Fig. 8, pretreatment of adipocytes with 100 μmol/L SP600125, resulted in a complete reversal of the antilipolytic effect of insulin, similar to the one obtained after pretreatment with 100 μmol/L DMAP. Whereas 100 μmol/L SP600125 completely blocked the inhibition of lipolysis by insulin, 100 μmol/L SP600125 only inhibited insulin-induced lipogenesis and glucose uptake down to 90% and 74% of the of the original response respectively. Again, these results are similar to the ones obtained using corresponding concentrations of DMAP.

Taken together, our results suggest a possible role for JNK in metabolic actions of insulin, especially in the case of antilipolysis. DMAP-mediated inhibition of JNK may therefore contribute to the inhibitory effects of DMAP on the three metabolic insulin responses investigated

4. Discussion

In this article we demonstrate new effects of the kinase inhibitor DMAP on metabolic insulin signaling pathways in rat adipocytes. Similar results were also obtained in human adipocytes (antilipolysis and lipogenesis, unpublished data), although the effects sometimes required higher concentration of the inhibitor. Full understanding of the mechanisms involved in the action of DMAP needs further investigation, but JNK could be one of its targets in cells, the inhibition of which may explain the effects of DMAP on metabolic signaling in adipocytes.

The different biological responses were affected somewhat differently by DMAP. The ability of insulin to counteract lipolysis was not inhibited by 10 μmol/L DMAP but was on the other hand completely reversed in the presence of 100 μmol/L inhibitor. This rapid and total effect is in contrast to the impact of DMAP on lipogenesis and glucose uptake which was weaker and more gradual, with a small inhibition at 10 μmol/L and a submaximal effect at 100 μmol/L. These differences may reflect different mechanisms whereby DMAP acts on the three signaling pathways. The rate of lipogenesis is to a large extent determined by the rate of glucose uptake into the cells. Hence, the signaling pathways leading to lipogenesis and glucose uptake are partly the same. This is in line with the similar effects DMAP had on these two responses to insulin.

Our results show that DMAP can function as a weak direct PKB inhibitor (Fig. 5d). However, the degree of

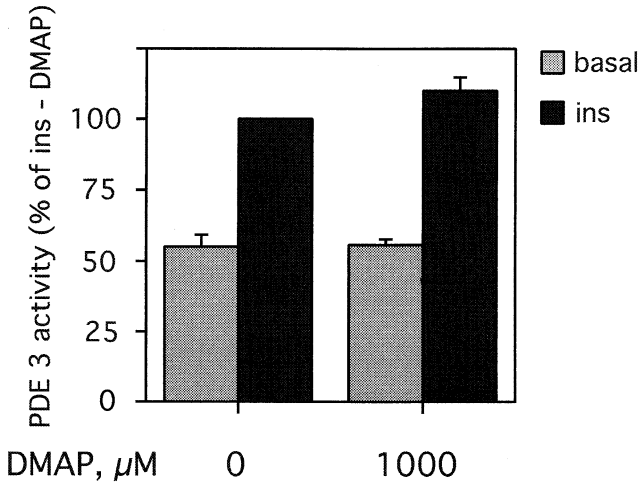

Fig. 6. Activation of PDE 3B in response to insulin. Primary rat adipocytes were preincubated for 30 minutes with or without 1 mmol/L of DMAP, and stimulated with or without 10 nmol/L insulin (ins) for 10 minutes. Membrane fractions were prepared and assayed for PDE 3 activity. Results are presented as percentage of the value obtained with insulin in the absence of DMAP and are means ± SD of two independent experiments. The absolute value for insulin-induced PDE 3 activity (taken as 100%) is 163–195 pmol cAMP/min/mL PCV.

inhibition of PKB by DMAP in cells did not correlate with the degree of the effects of DMAP on the biological responses. For example, at 100 μmol/L DMAP the ability of insulin to counteract lipolysis was completely abrogated (Fig. 1), whereas insulin-induced PKB activity in cells was not affected (Fig. 5a, Fig. 6). Furthermore, at 1 mmol/L DMAP, insulin-induced lipogenesis was inhibited down to 10% of the original insulin-induced response, whereas 70% of the PKB activity was intact. Our conclusion is therefore that PKB is not likely to be a functional target for DMAP in cells.

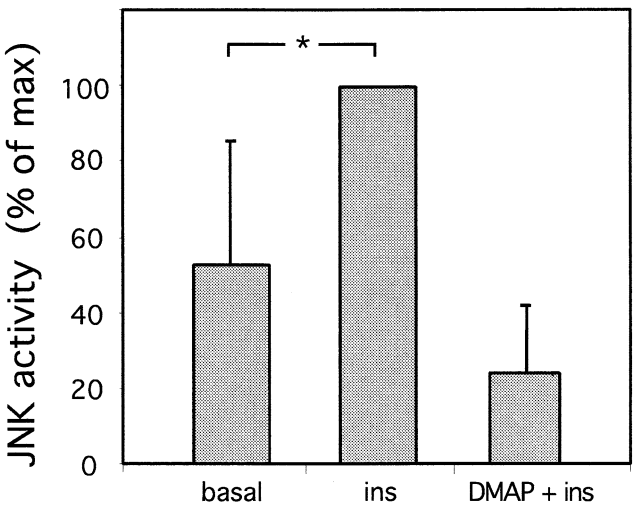

Fig. 7. Inhibition of insulin-induced JNK activity by DMAP. Primary rat adipocytes were preincubated for 30 minutes with 1 mmol/L of DMAP and stimulated with 10 nmol/L of insulin (ins) for 1 minute. JNK activity was measured in cytosol fractions as kinase activity towards ATF2 in JNK1-immunoprecipitates. Results are presented as percentage of the maximal value, and are means ± SD of two to six experiments. *$P < 0.05$

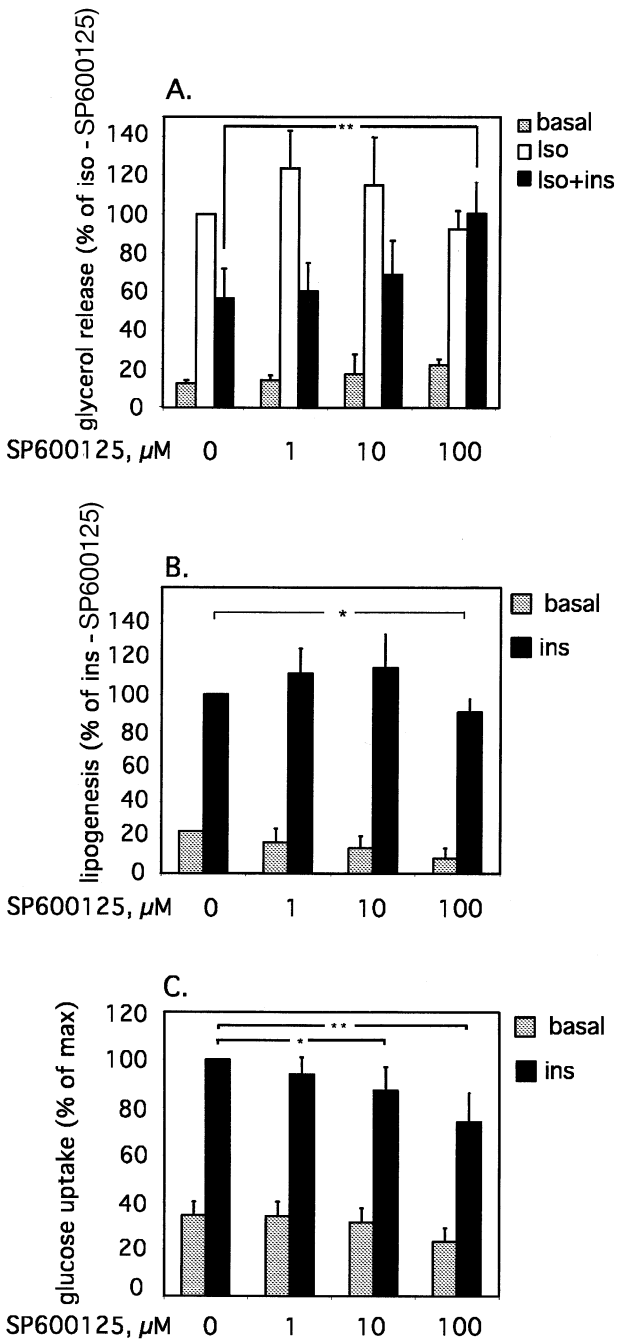

Fig. 8. Possible role for JNK in metabolic actions of insulin Primary rat adipocytes were preincubated for 30 minutes with or without different concentrations of SP600125 (SP), and then stimulated with or without 100 nmol/L isoprenalin (iso) (a) and with or without 1 nmol/L (ins) (a, b, c) for 30 minutes. Lipolysis (a), lipogenesis (b), and glucose uptake (c) was then measured as accumulated glycerol release (a), and uptake of ^{3}H-glucose (b), and 2-deoxy ^{3}H –glucose (c) respectively. Results are presented as percent of the value obtained with isoprenalin (a) or insulin (b, c) in the absence of SP600125, and are mean values ± SD from four (a, b) and five (c) independent experiments. *$P < 0.05$, **$P < 0.01$. ***$P < 0.001$. The absolute values for the isoprenalin-induced lipolysis (taken as 100%) in (a) are 305–475 nmol glycerol/30 min/50 μL PCV.

In our experimental setting, a 30% PKB inhibition did not influence insulin-induced activation of PDE 3B in rat adipocytes (Fig. 6). This was somewhat surprising since

there is now accumulating evidence that PKB can act as a PDE kinase both *in vitro* and *in vivo*. For example, Wijkander et al. have shown that PDE kinase activity from rat adipocytes co-elutes with PKB from Mono Q and Superdex columns [6]. Furthermore, immunoprecipitated as well as recombinant PKB can be used to phosphorylate PDE 3B *in vitro* [7,8]. A role for PKB as a PDE kinase *in vivo* has been established by Ahmad et al. [7] and Kitamura et al. [8] in FDCP2 cells and 3T3 L1 adipocytes respectively, by detecting downstream effects on PDE 3B in cells overexpressing different mutants of PKB and PDE 3B. Most likely, the remaining 70% of PKB activity is enough to fully activate PDE 3B.

Since PKB inhibition did not provide a satisfying explanation to the effects of DMAP on the metabolic actions of insulin, we wanted to explore which other targets of DMAP could play a role. DMAP has previously been used as an inhibitor of JNK activation by TNFα [28,29], and in this article we show that DMAP also blocks the activation of JNK induced by insulin. How this inhibition is mediated is not known, although one group suggests upregulated expression of a JNK phosphatase as a possible mechanism [29]. To study the possible involvement of JNK in the mechanisms whereby DMAP exerts its effects on insulin-induced antilipolysis, lipogenesis, and glucose uptake, we used SP600125, an anthrapyrazolone inhibitor of JNK [36]. Pretreatment of adipocytes with SP600125 completely reversed the antilipolytic action of insulin, and partly inhibited lipogenesis and glucose uptake, suggesting a role for JNK in the signaling pathways leading to these responses. SP600125 was described in 2001 by Bennett et al. [36], who reported SP600125 to inhibit JNK1 *in vitro* with an IC50 value of 0.04 μmol/L, and with a more than 20-fold selectivity versus a range of other kinases, for example PKA (IC50 $>10\mu$mol/L) and PKB (IC50 1 μmol/L). The concentration required for full effect in cells was 25–50 μmol/L. SP600125 has since been relatively widely used in studies of various apoptotic or mitogenic events. During the preparation of this manuscript the specificity of SP600125 was however questioned by Bain et al. [37], who showed that additional kinases, for example p70 ribosomal protein S6 kinase, glucocorticoid-induced kinase, and AMP-activated protein kinase was inhibited by SP600125 *in vitro*. Consequently, it should be noted that, before further studies have been carried out to fully investigate the effects of this inhibitor in intact cells, data should be interpreted with caution.

Where in the antilipolytic pathway JNK may be acting remains unclear so far. Neither PKB nor PDE 3B activation in response to insulin was affected by SP600125 (data not shown), suggesting that JNK possibly acts downstream of PDE 3B, close to the biological target HSL. For example 5′-AMP dependent protein kinase (AMPK), a negative regulator of HSL [38,39], has been shown to be activated in response to arsenite [40], a strong activator of JNK, providing a possible link between JNK/arsenite signaling, and lipolysis.

As discussed in the Introduction, the signal transduction pathways leading to increased lipogenesis and glucose uptake are less well understood, but have been suggested to involve PKB. The exact role of JNK in these responses remains to be established.

It should be emphasized that, even if DMAP-inhibition of JNK might explain some of the *in vivo* effects of DMAP, these are most likely not the only targets of DMAP in the cells. Hence, the main target, inhibition of which mediates the effects of DMAP on insulin signaling, may remain to be identified.

Considering the data presented here, DMAP has proved to be a rather promiscuous agent that should be used with care. To identify further the targets mediating its effects will be a future challenge. The relatively high concentration needed to obtain maximal effects in cells might also pose a problem. However, defect metabolic signaling is potentially of great importance for the development of common disorders such as obesity and insulin resistance, and the possibility to manipulate such pathways is therefore of great therapeutic interest. Bearing in mind the role of adipose tissue in insulin resistant disorders [41], it might be of interest to further develop pluripotent drugs acting on lipolysis as well as glucose metabolism in fat cells.

An interesting finding was that JNK might be involved in mediating metabolic responses to insulin, especially the antilipolytic effect of insulin. To confirm this role for JNK, to establish where in the signaling cascade JNK acts and to evaluate the biological relevance of this observation, will be important issues in the future.

In conclusion, in this article we show that DMAP inhibits insulin-induced antilipolysis, lipogenesis, and glucose uptake in primary rat adipocytes. Two of these biological responses were affected already at 10 μmol/L of the inhibitor. The mechanism for this inhibition is still not fully elucidated, but JNK could be the functional cellular target for DMAP in cells. In addition, we have identified a new possible role for JNK as a component of the antilipolytic signaling pathway of insulin.

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References

[1] Eriksson H, Ridderstråle M, Degerman E, Ekholm D, Smith CJ, Manganiello VC, Belfrage P, Tornqvist H. Evidence for the key role of the adipocyte cgmp-inhibited camp phosphodiesterase in the antilipolytic action of insulin. Biochim Biophys Acta 1995;1266: 101–7.

[2] Manganiello VC, Smith CJ, Degerman E, Vasta V, Tornqvist H, Belfrage P. Molecular mechanisms involved in the antilipolytic action of insulin: phosphorylation and activation of a particulate adipocyte camp phosphodiesterase. Adv Exp Med Biol 1991;293: 239–48.

[3] Rahn T, Ridderstråle M, Tornqvist H, Manganiello V, Fredrikson G, Belfrage P, Degerman E. Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cgmp-inhibited camp phosphodiesterase in rat adipocytes. Studies using the selective inhibitor wortmannin. FEBS Lett 1994;350:314–8.

[4] Degerman E, Smith CJ, Tornqvist H, Vasta V, Belfrage P, Manganiello VC. Evidence that insulin and isoprenaline activate the cgmp-inhibited low-km camp phosphodiesterase in rat fat cells by phosphorylation. Proc Natl Acad Sci USA 1990;87:533–7.

[5] Holm C, Osterlund T, Laurell H, Contreras JA. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. Annu Rev Nutr 2000;20:365–93.

[6] Wijkander J, Landström TR, Manganiello V, Belfrage P, Degerman E. Insulin-induced phosphorylation and activation of phosphodiesterase 3b in rat adipocytes: possible role for protein kinase b but not mitogen-activated protein kinase or p70 s6 kinase. Endocrinology 1998;139:219–27.

[7] Ahmad F, Cong LN, Stenson Holst L, Wang LM, Rahn Landström T, Pierce JH, Quon MJ, Degerman E, Manganiello VC. Cyclic nucleotide phosphodiesterase 3b is a downstream target of protein kinase b and may be involved in regulation of effects of protein kinase b on thymidine incorporation in fdcp2 cells. J Immunol 2000;164:4678–88.

[8] Kitamura T, Kitamura Y, Kuroda S, Hino Y, Ando M, Kotani K, Konishi H, Matsuzaki H, Kikkawa U, Ogawa W, Kasuga M. Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3b by the serine-threonine kinase akt. Mol Cell Biol 1999; 19:6286–96.

[9] Göransson O, Resjö S, Rönnstrand L, Manganiello V, Degerman E. Ser-474 is the major target of insulin-mediated phosphorylation of protein kinase b beta in primary rat adipocytes. Cell Signal 2002;14: 175–82.

[10] Shepherd PR, Withers DJ, Siddle K. Phosphoinositide 3-kinase: the key switch mechanism in insulin signaling. Biochem J 1998;333: 471–90.

[11] Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. J Clin Invest 2000;106:165–9.

[12] Kohn AD, Summers SA, Birnbaum MJ, Roth RA. Expression of a constitutively active akt ser/thr kinase in 3t3-l1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 1996;271:31372–8.

[13] Tanti JF, Grillo S, Gremeaux T, Coffer PJ, Van Obberghen E, Le Marchand-Brustel Y. Potential role of protein kinase b in glucose transporter 4 translocation in adipocytes. Endocrinology 1997;138: 2005–10.

[14] Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI, Quon MJ. Physiological role of akt in insulin-stimulated translocation of glut4 in transfected rat adipose cells. Mol Endocrinol 1997;11:1881–90.

[15] Smith U, Carvalho E, Mosialou E, Beguinot F, Formisano P, Rondinone C. Pkb inhibition prevents the stimulatory effect of insulin on glucose transport and protein translocation but not the antilipolytic effect in rat adipocytes. Biochem Biophys Res Commun 2000;268: 315–20.

[16] Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase akt2 (pkb beta). Science 2001;292:1728–31.

[17] Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M, Matsumoto M, Maeda T, Konishi H, Kikkawa U, Kasuga M. Requirement for activation of the serine-threonine kinase akt (protein kinase b) in insulin stimulation of protein synthesis but not of glucose transport. Mol Cell Biol 1998;18:3708–17.

[18] Neant I, Guerrier P. 6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity. Exp Cell Res 1988;176:68–79.

[19] Rime H, Neant I, Guerrier P, Ozon R. 6-Dimethylaminopurine (6-dmap), a reversible inhibitor of the transition to metaphase during the first meiotic cell division of the mouse oocyte. Dev Biol 1989;133: 169–79.

[20] Szollosi MS, Kubiak JZ, Debey P, de Pennart H, Szollosi D, Maro B. Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. J Cell Sci 1993;104:861–72.

[21] Davis RJ. Signal transduction by the jnk group of map kinases. Cell 2000;103:239–52.

[22] Kim SJ, Kahn CR. Insulin stimulates phosphorylation of c-jun, c-fos, and fos-related proteins in cultured adipocytes. J Biol Chem 1994; 269:11887–92.

[23] Morino K, Maegawa H, Fujita T, Takahara N, Egawa K, Kashiwagi A, Kikkawa R. Insulin-induced c-jun n-terminal kinase activation is negatively regulated by protein kinase c delta. Endocrinology 2001; 142:2669–76.

[24] Standaert ML, Bandyopadhyay G, Antwi EK, Farese RV. Ro 31-8220 activates c-jun n-terminal kinase and glycogen synthase in rat adipocytes and l6 myotubes. Comparison to actions of insulin. Endocrinology 1999;140:2145–51.

[25] Lee YH, Giraud J, Davis RJ, White MF. C-jun n-terminal kinase (jnk) mediates feedback inhibition of the insulin signaling cascade. J Biol Chem 2003;278:2896–902.

[26] Moxham CM, Tabrizchi A, Davis RJ, Malbon CC. Jun n-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin in vivo. J Biol Chem 1996;271:30765–73.

[27] Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-jun nh(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of ser(307). J Biol Chem 2000;275:9047–54.

[28] Marino MW, Dunbar JD, Wu LW, Ngaiza JR, Han HM, Guo D, Matsushita M, Nairn AC, Zhang Y, Kolesnick R, Jaffe EA, Donner DB. Inhibition of tumor necrosis factor signal transduction in endothelial cells by dimethylaminopurine. J Biol Chem 1996;271:28624–9.

[29] De Cesaris P, Starace D, Starace G, Filippini A, Stefanini M, Ziparo E. Activation of jun n-terminal kinase/stress-activated protein kinase pathway by tumor necrosis factor alpha leads to intercellular adhesion molecule-1 expression. J Biol Chem 1999;274:28978–82.

[30] Rydén M, Dicker A, van Harmelen V, Hauner H, Brunnberg M, Perbeck L, Lönnqvist F, Arner P. Mapping of early signaling events in tumor necrosis factor-alpha–mediated lipolysis in human fat cells. J Biol Chem 2002;277:1085–91.

[31] Rodbell M. Metabolism of isolated fat cells. J Biol Chem 1964;239: 375–80.

[32] Honnor RC, Dhillon GS, Londos C. Camp-dependent protein kinase and lipolysis in rat adipocytes. I. Cell preparation, manipulation, and predictability in behavior. J Biol Chem 1985;260:15122–9.

[33] Wieland O. Eine enzymatische methode zur bestimmung von glycerin. Biochem Z 1957;239:313-9.

[34] Moody AJ, Stan MA, Stan M, Gliemann J. A simple free fat cell bioassay for insulin. Horm Metab Res 1974;6:12–6.

[35] Tanti JF, Cormont M, Gremeaux T, Le Marchand-Brustel Y. Assays of glucose entry, glucose transporter amount, and translocation. Methods Mol Biol 2001;155:157–65.
[36] Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. Sp600125, an anthrapyrazolone inhibitor of jun n-terminal kinase. Proc Natl Acad Sci USA 2001; 98:13681–6.
[37] Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. Biochem J 2003;371:199–204.
[38] Sullivan JE, Brocklehurst KJ, Marley AE, Carey F, Carling D, Beri RK. Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with aicar, a cell-permeable activator of amp-activated protein kinase. FEBS Lett 1994;353:33–6.
[39] Garton AJ, Campbell DG, Carling D, Hardie DG, Colbran RJ, Yeaman SJ. Phosphorylation of bovine hormone-sensitive lipase by the amp-activated protein kinase. A possible antilipolytic mechanism. Eur J Biochem 1989;179:249–54.
[40] Corton JM, Gillespie JG, Hardie DG. Role of the amp-activated protein kinase in the cellular stress response. Curr Biol 1994;4:315–24.
[41] Large V, Arner P. Regulation of lipolysis in humans. Pathophysiological modulation in obesity, diabetes and hyperlipidaemia. Diabetes Metab 1998;24:409–18.