The handle http://hdl.handle.net/1887/35740 holds various files of this Leiden University dissertation

**Author:** Heemskerk, Mattijs  
**Title:** The role of energy & fatty acid metabolism in obesity and insulin resistance  
**Issue Date:** 2015-10-06
Long term niacin treatment induces insulin resistance and adrenergic responsiveness in adipocytes by adaptive down-regulation of phosphodiesterase 3B

Mattijs M. Heemskerk*, Sjoerd A.A. van den Berg*, Amanda C.M. Pronk, Jan-Bert van Klinken, Mariëtte R. Boon, Louis M. Havekes, Patrick C.N. Rensen, Ko Willems van Dijk, Vanessa van Harmelen

*Both authors contributed equally

AJP Endocrinology & Metabolism 2014, 306(7): 808-813
**Abstract**

The lipid lowering effect of niacin has been attributed to the inhibition of cAMP production in adipocytes, thereby inhibiting intracellular lipolysis and release of non-esterified fatty acids (NEFA) to the circulation. However, long term niacin treatment leads to a normalization of plasma NEFA levels and induces insulin resistance, for which the underlying mechanisms are poorly understood. The current study addressed the effects of long term niacin treatment on insulin-mediated inhibition of adipocyte lipolysis and focused on the regulation of cAMP levels. APOE*3-Leiden.CETP transgenic mice treated with niacin for 15 weeks were subjected to an insulin tolerance test and showed whole body insulin resistance. Similarly, adipocytes isolated from niacin treated mice were insulin resistant and, interestingly, exhibited an increased response to cAMP stimulation by 8Br-cAMP, β₁, and β₂-adrenergic stimulation. Gene expression analysis of the insulin and β-adrenergic pathways in adipose tissue indicated that all genes were down-regulated, including the gene encoding the cAMP degrading enzyme phosphodiesterase 3B (PDE3B). In line with this, we showed that insulin induced a lower PDE3B response in adipocytes isolated from niacin treated mice. Inhibiting PDE3B with cilostazol increased lipolytic responsiveness to cAMP stimulation in adipocytes. These data show that long term niacin treatment leads to a down-regulation of PDE3B in adipocytes which could explain part of the observed insulin resistance and the increased responsiveness to cAMP stimulation.
**Introduction**

Niacin, also known as vitamin B₃, is required for the synthesis of the co-factor nicotinamide adenine dinucleotide (NAD⁺) and is therefore essential for oxidative phosphorylation in energy metabolism [1]. It has been used for more than 50 years for the treatment of dyslipidemias, as it decreases plasma triglycerides, LDL-cholesterol and hepatic VLDL triglyceride production [2], in addition to increasing HDL-cholesterol. Supplementation with niacin was shown to decrease risk of cardiovascular disease and atherosclerosis in dyslipidemic humans [3] and in dyslipidemic mouse models [4], using the APOE*3-Leiden.cholesteryl ester transfer protein (CETP) transgenic female mouse.

The molecular mechanism by which niacin conveys its lipid-lowering effects is mostly unknown. The receptor for niacin, HCA₂ (formerly known as GPR109A) has been shown to play an important role in acute anti-lipolytic effects [5, 6], but is not required for the long term lipid-lowering effects [6]. This receptor is expressed mostly in spleen, immune cells and adipose tissue [7] where binding of niacin leads to the release of a Gα-inhibitory subunit that inhibits production of the secondary messenger cyclic AMP by adenyl cyclase (AC). Niacin is known to inhibit lipolysis in adipocytes via this reduction of cAMP [8], which leads to a reduced protein kinase A (PKA) activation and thus less phosphorylation and activation of the lipolytic enzyme hormone sensitive lipase (HSL) [9]. By inhibiting lipolysis, less NEFAs are released by adipocytes thus making less substrate available for VLDL-Triglyceride (TG) production in the liver. Niacin may therefore lower lipid levels via its anti-lipolytic effect. However, during niacin administration the initial drop in NEFA and glycerol is followed within hours by a rebound and normalization of NEFA and glycerol levels and adipose tissue lipolysis rates [2, 6, 10-12].

In addition, long term niacin administration leads to insulin resistance in liver, adipose and muscle tissue [13, 14] for which the underlying mechanisms are poorly understood. In adipocytes, binding of insulin to its receptor leads to phosphorylation and activation of protein kinase B (PKB/Akt) and phosphodiesterase 3B, breaking down cAMP which in turn reduces NEFA release from the cells.

We hypothesized that long term niacin treatment modulates insulin signaling by acting at the level of PDE3B. Oh *et al.* [10] have previously shown that 24h treatment with niacin induces insulin resistance and reduces expression of PDE3B in adipose tissue, hinting towards a possible role for PDE3B. In this study we functionally tested in APOE*3-Leiden.CETP transgenic female mice whether long term niacin-induced down regulation of the enzyme PDE3B would lead to less PDE3B activity when stimulated by insulin, which would explain (part of) the observed insulin resistance in adipose tissue.
**Materials and Methods**

**Animal model**

In contrast to wildtype mice, female APOE*3-Leiden.CETP mice \[15\] have a human-like lipoprotein profile, which makes them exquisitely suited to test hypolipidemic drugs, such as niacin \[4\]. This mouse model was bred at the Leiden University Medical Center. At age 15 ± 1 week, mice were fed a western type diet (Diet T with 0.1% cholesterol, which consisted of 17 kcal% protein, 43 kcal% carbohydrate and 41 kcal% fat; AB Diets, Woerden, the Netherlands) with or without niacin (0.3% w/w, Sigma Aldrich, St Louis, MO, USA). Body weight was registered weekly. Animals were housed in a controlled environment (21°C, 40-50% humidity) with a daily 12h photoperiod (07h00-19h00). Food and tap water were available *ad libitum* during the whole experiment. All experiments were performed after a 15 week dietary intervention period. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare. The institutional ethics committee for animal procedures from the Leiden University Medical Center, Leiden, The Netherlands approved the protocols.

**Intraperitoneal insulin tolerance test**

Food was withdrawn from all animals at 08h00 for a period of 6.5 hours. Subsequently, all animals received a single intra-peritoneal (*ip*) injection with insulin (0.2 U/kg, Novo-Nordisk, Bagsværd, Denmark) and blood samples were taken every 30 min for a period of 2 hours. Blood samples were taken from the tail tip into chilled paraoxon-coated capillaries and placed on ice to prevent *ex vivo* lipolysis.

**Organ collection**

All animals were sacrificed and organs were collected in the fed state between 08h00 and 9h30 unless otherwise indicated. Blood was collected by cardiac puncture and plasma was collected after centrifugation. Fresh subcutaneous (sW AT), gonadal (gW AT) and visceral (vW AT) white adipose tissue were harvested and used for determination of morphometry and lipolysis experiments as described below. In addition, a portion of the gW AT was frozen for qPCR and cAMP measurement. Liver and adrenal glands were harvested quantitatively and weighed prior to freezing. All tissues were stored at -80°C prior to further analysis.

**Plasma and liver parameters**

Commercially available kits were used to determine plasma levels of triglycerides (1488872, Roche Molecular Biochemicals, Indianapolis, IL, USA), total cholesterol (236691 Roche
Molecular Biochemicals), phospholipids (Instruchemie, Delftzijl, The Netherlands), NEFAs (Wako Chemicals, Neuss, Germany), glucose (Accuchek, Roche, The Netherlands) and insulin (Crystal Chem Inc., Downers Grove, IL, USA), according to the manufacturer's instructions.

**Adipocyte morphometry**

Adipose tissue from the gW AT, sW AT and vW AT were minced and digested in 0.5 g/l collagenase in HEPES buffer (pH 7.4) with 20 g/l of dialyzed bovine serum albumin (BSA, fraction V, Sigma Aldrich) for 1 h at 37°C. The disaggregated WAT was filtered through a nylon mesh with a pore size of 236 μm. For the isolation of mature adipocytes, cells were obtained from the surface of the filtrate and washed several times. Cell size and volume of mature adipocytes was determined from micrographs (± 1000 cells/WAT sample) using image analysis software that was developed in-house in MATLAB (MathWorks, Natick, MA, USA).

**Lipolysis experiments in isolated adipocytes**

Adipocytes were incubated in DMEM/F12 with 2% (w/w) BSA in 96 well plates with ~10,000 adipocytes in 200 μl per well in the presence of #1) No additional reagents, #2) 10⁻⁷ M of the PDE3B sensitive cAMP analogue 8Bromo-cAMP, #3) 10⁻³ M 8Br-cAMP + 10⁻¹⁰ M insulin (Novo-Nordisk), #4) 10⁻⁶ M of the selective β₂-adrenergic agonist terbutaline, #5) 10⁻⁶ M of the selective β₁-adrenergic agonist dobutamine. The wells of the adipocytes from niacin treated mice contained 10⁻⁶ M niacin, while control adipocyte medium did not contain niacin. Basal lipolysis rates were determined in incubations without these reagents. PDE3B responsiveness in adipocytes from control and niacin treated mice was assayed by lipolysis assay using the selective PDE3 inhibitor cilostazol (all obtained from Sigma Aldrich). under the following additional conditions: #6-8) 10⁻⁶ or 10⁻⁵ or 10⁻⁴ M cilostazol, #9-11) 10⁻³ M 8Br-cAMP + 10⁻⁶ or 10⁻⁵ or 10⁻⁴ M cilostazol. The adipocytes were incubated for two hours at 37°C, after which 100 μl of lipolysis assay medium was frozen at -20°C. At the day of analysis, 10 μl of lipolysis assay medium was mixed with 100 μl of Free Glycerol Reagent (F6428, Sigma Aldrich) and 0.5μl Amplex UltraRed Reagent (A36006, Invitrogen, Carlsbad, CA, USA). After 10 min of incubation in the dark, the fluorescence was excited at 530 nm and measured at 590 nm on a Fluorometer (SpectraMAX Gemini, Molecular Devices, CA, USA).

**Adipose tissue cAMP measurement**

cAMP concentrations were measured in 50 mg of gWAT, using the cAMP direct immunoassay kit (Biovision, Inc, Milpitas, CA, USA).
Quantitative PCR

RNA was isolated from gWAT using the Nucleospin RNA/Protein kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Subsequently, 1μg of RNA was used for cDNA synthesis by iScript (BioRad, Hercules, CA, USA), which was purified by the Nucleospin Gel and PCR clean-up kit (Machery Nagel). Real-Time PCR was carried out on the IQ5 PCR machine (BioRad) using the Sensimix SYBR Green RT-PCR mix (Quanta, London, UK) and QuantiTect SYBR Green RT-PCR mix (Qiagen, Venlo, the Netherlands). Target mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA levels. Primer sequences are listed in table 1.

Table 1: Mouse qPCR primer sequences all with an optimal temperature at 60°C.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsl</td>
<td>AGACACCAGCCAACGGATAC</td>
<td>ATCACCCTCGAAGAAGCA</td>
</tr>
<tr>
<td>Insr</td>
<td>ATGGGCTTGGGAAGAGAT</td>
<td>GGATGTCATACCCAGGAC</td>
</tr>
<tr>
<td>Irs1</td>
<td>CGATGGCTTCTCAACGCTG</td>
<td>CAGCCCCGCTGTGATGTTG</td>
</tr>
<tr>
<td>Pde3b</td>
<td>AAGGCCACCGGAGTACTAT</td>
<td>CACACTGCTCAAGTCCAG</td>
</tr>
<tr>
<td>Adrb1</td>
<td>TCCTACCCAGAGTTCCGGT</td>
<td>GGCACTGAGGAAGAGAC</td>
</tr>
<tr>
<td>Adrb2</td>
<td>AAGACGACAGGACTTCTTGCT</td>
<td>GCACACCCAAGGAGATAT</td>
</tr>
<tr>
<td>Adrb3</td>
<td>TGAAACAGCGACAGGACA</td>
<td>AGTCAGTCAAGCGTCCC</td>
</tr>
<tr>
<td>Arrb1</td>
<td>CCCGCTTTCCCAGGAGAC</td>
<td>AAGGACAGGAGTTCAAG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GGAGCTGCTGCTCTCAAA</td>
<td>TTGCTCAGTGTCCTGCTGG</td>
</tr>
</tbody>
</table>

Statistics

All data in figures are represented as mean ± standard error of the mean. The mean of all data was tested between groups for differences by unpaired T-Test for normally distributed data. A repeated measures ANOVA was used when indicated. Threshold for significance was set at 5%. Tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).
Results

Niacin does not alter body composition

We have applied a humanized mouse model, which has previously been shown [4] to react to niacin on plasma lipid parameters, atherosclerosis and body weight in a similar manner as dyslipidemic humans. After fifteen weeks of niacin treatment body weight (Table 2) and weight gain (not shown) did not differ between the niacin treated and control mice. Adipose tissue depots were similar in weight, whereas the adrenal glands were heavier after niacin treatment (Table 2). Liver cholesterol content was significantly lower in niacin treated mice (Table 2).

Niacin lowers plasma lipids, but increases fasting glucose and insulin

Long term niacin treatment resulted in the expected decrease of non-fasting plasma total cholesterol, triglycerides and phospholipids (Table 2). In contrast to short term niacin treatment, long term niacin treatment did not lower NEFA levels [2], but slightly lowered glycerol levels as compared to control mice (Table 2). Glucose and insulin concentrations were significantly higher after niacin treatment (Table 2), suggesting insulin resistance.
Table 2: Body composition, liver and plasma metabolic parameters and adipocyte sizes of mice treated 15 weeks with or without 0.3% niacin in the diet. Fasting values were determined after 6.5h without feeding from 08h00 until 14h30. *p≤0.05, **p≤0.01.

<table>
<thead>
<tr>
<th></th>
<th>Control Mean±SD</th>
<th>Niacin Mean±SD</th>
<th>N</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body composition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28.9±2.6</td>
<td>29.3±5.3</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>Gonadal adipose tissue (g)</td>
<td>0.48±0.19</td>
<td>0.54±0.34</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>Visceral adipose tissue (g)</td>
<td>0.40±0.11</td>
<td>0.48±0.28</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>Subcutaneous adipose tissue (g)</td>
<td>0.32±0.09</td>
<td>0.39±0.23</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>Adrenal glands (mg)</td>
<td>3.9±1.2</td>
<td>5.1±1.0</td>
<td>13</td>
<td>*</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1.44±0.34</td>
<td>1.28±0.24</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Liver lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (nmol/mg protein)</td>
<td>163±67</td>
<td>96±29</td>
<td>13</td>
<td>**</td>
</tr>
<tr>
<td>Triglycerides (nmol/mg protein)</td>
<td>851±382</td>
<td>651±213</td>
<td>13</td>
<td>0.11</td>
</tr>
<tr>
<td>Phospholipids (nmol/mg protein)</td>
<td>165±42</td>
<td>148±23</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Plasma lipids, glucose and insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed state cholesterol (mM)</td>
<td>13.0±4.9</td>
<td>5.1±1.4</td>
<td>13</td>
<td>**</td>
</tr>
<tr>
<td>Fed state triglycerides (mM)</td>
<td>4.11±2.66</td>
<td>0.94±0.19</td>
<td>13</td>
<td>**</td>
</tr>
<tr>
<td>Fed state phospholipids (mM)</td>
<td>4.02±0.88</td>
<td>2.94±0.53</td>
<td>13</td>
<td>**</td>
</tr>
<tr>
<td>Fasting NEFA (mM)</td>
<td>1.1±0.3</td>
<td>1.0±0.2</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting glycerol (mM)</td>
<td>0.92±0.29</td>
<td>0.74±0.14</td>
<td>12</td>
<td>0.08</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>9.1±0.9</td>
<td>10.6±0.9</td>
<td>12</td>
<td>*</td>
</tr>
<tr>
<td>Fasting insulin (ng/ml)</td>
<td>0.49±0.21</td>
<td>0.82±0.39</td>
<td>12</td>
<td>*</td>
</tr>
<tr>
<td><strong>Adipocyte mean volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonadal (pl)</td>
<td>342±98</td>
<td>334±140</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>Visceral (pl)</td>
<td>152±39</td>
<td>190±97</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>Subcutaneous (pl)</td>
<td>167±43</td>
<td>158±60</td>
<td>13</td>
<td>NS</td>
</tr>
</tbody>
</table>

Niacin induces insulin resistance in vivo

To verify the development of in vivo insulin resistance upon long term niacin treatment we performed an insulin tolerance test. Insulin stimulates glucose uptake in muscle and adipose tissue and suppresses WAT glycerol release. After an ip injection of insulin, blood was drawn and at indicated times glucose (Fig. 1A), glycerol (Fig. 1B) and insulin (Fig. 1C) were measured. Glucose levels dropped and insulin levels rose in both groups. As glucose levels were already higher due to niacin treatment at t=0, the percentage change in glucose, glycerol and insulin levels from the t=0 values were calculated (data not shown). A repeated measures ANOVA of the percentage change in insulin values indicated no significant effect of niacin, while a similar analysis of glucose values showed significant treatment differences. At
60 min, the percentage change in glucose was smaller for niacin treated animals, indicating insulin resistance on a whole body level. Insulin induced a decrease in glycerol levels in the control group, but failed to do so in the niacin treated group, indicating adipose tissue insulin resistance.

![Graphs showing changes in glucose, glycerol, and insulin levels over time for control and niacin-treated groups.](image)

Figure 1: Intraperitoneal insulin tolerance tests of mice treated with or without niacin. After 0.2U/kg insulin injection A) glucose, B) glycerol and C) insulin concentrations were measured during 2 h. All values are means±SEM. N=12 mice per group, *p<0.05 compared to control treatment, **p<0.01 compared to niacin treatment, #p<0.05 compared to niacin treatment, when calculated as percentage change from t=0 level.
Niacin reduces the anti-lipolytic effect of insulin in isolated adipocytes

To study whether long term niacin treatment also alters the response to insulin at the level of adipocyte lipolysis, we isolated gWAT adipocytes from control and niacin treated mice and performed an ex vivo lipolysis assay. Basal lipolysis (i.e. unstimulated lipolysis) did not differ between the niacin treated and control mice (Fig. 2A). 8Br-cAMP stimulated glycerol release was higher in adipocytes from niacin treated mice compared to control treatment. The anti-lipolytic effect of insulin, reflected by the percentage lipolysis suppression by insulin of 8Br-cAMP stimulated lipolysis, was significantly smaller in the niacin treated adipocytes (Fig. 2B). This implies a reduced insulin response of adipocytes from niacin treated mice.

Niacin increases the lipolytic effect of β-adrenergic stimulation in isolated adipocytes

Similar to testing the response of adipocyte lipolysis to inhibition by insulin, β₁ and β₂-adrenergic stimulation of gonadal adipocyte lipolysis was tested. Gonadal adipocytes from niacin treated mice exhibited a greater response to both the β₁-agonist dobutamine and the β₂-agonist terbutaline, as well as the secondary lipolytic messenger 8Br-cAMP (Fig 2A). These data show that lipolysis in niacin treated adipocytes is more responsive to β-adrenergic and cAMP stimulation.
Figure 2: Isolated adipocyte responsiveness to lipolysis stimulation and inhibition. A) Glycerol concentration in ex vivo gonadal adipocyte medium after 2 h incubation with different reagents. B) Suppression of gonadal adipocyte lipolysis by 10^{-8}M insulin (100 – 100*(8Br-cAMP+Ins / 8Br-cAMP)). C) gWAT mRNA fold change of niacin treatment compared to control treatment. Insr (Insulin receptor), Irs1 (Insulin receptor substrate 1), Pde3b (Phosphodiesterase 3B), Adrb1,2,3 (β-Adrenoceptor 1,2,3), Arrb1 (β-arrestin 1), Hsl (Hormone sensitive lipase). All values are means±SEM. N=13 mice per group, *p≤0.05, **p≤0.01, ***p≤0.001.

Niacin down-regulates genes involved in both the insulin and the β-adrenergic signaling pathways that regulate lipolysis

Expression of genes involved in the insulin and/or β-adrenergic pathways, were analyzed by qPCR in gWAT. The niacin treated adipose tissues showed a down-regulation of the insulin signaling cascade: mRNA levels of the insulin receptor (Insr), insulin receptor substrate-1 (Irs1) and Pde3b were down-regulated by 45%, 97% and 53%, respectively, compared to control (Fig. 2C). Also mRNA levels of the lipolytic enzyme hormone sensitive lipase (Hsl) were down-regulated (62%) in the niacin treated mice. Furthermore, all β-adrenergic receptor genes were significantly down-regulated. The β_1-adrenoceptor (Adrb1) was down-regulated
by 68%, Adrb2 by 74% and Adrb3 by 88%. The intracellular adrenergic adaptor β-Arrestin1 (Arrb1) was also down-regulated by 42%. These data indicate a decreased gene expression of the insulin and β-adrenergic receptors, and their post-receptor signaling pathways to lipolysis.

**Niacin treated adipocytes display less PDE3B capacity**

As the down-regulation of the β-adrenoceptors was not consistent with the observed increased β-adrenergic response, we investigated whether the increased β-adrenergic response was the result of post-receptor signaling changes. Therefore we first tested the basal levels of the lipolytic post-receptor mediator cAMP. The cAMP concentration of non-fasted gW AT showed no difference after long term niacin treatment (control: 19.9±6.5, niacin: 21.6±8.2 pmol/g adipose tissue, p=0.63).

We then focused on the post receptor enzyme PDE3B, catalyzing cAMP hydrolysis, whose gene expression was down-regulated after niacin treatment. PDE3B is the most predominant PDE3 isoform in adipose tissue [16] which is activated by insulin. The role of PDE3B in lipolysis was characterized in gWAT adipocytes using the PDE3 selective inhibitor cilostazol (Fig 3). Adding cilostazol to unstimulated adipocytes in basal medium did not increase lipolysis, indicating that under basal conditions PDE3B was not active. However, when 8Br-cAMP stimulated adipocytes were subjected to increasing concentrations of cilostazol, there was a sharp rise in lipolysis. Therefore, inhibiting PDE3B capacity can increase the responsiveness to cAMP stimulation, e.g. by catecholamines. Furthermore, lipolysis of adipocytes from niacin treated mice was stimulated to a lesser degree by 10^{-4} M cilostazol than control treated mice. A repeated measures ANOVA indicated a significant interaction (p<0.0001) between the concentration of cilostazol and the 8Br-cAMP stimulated control/niacin adipocytes. This implied a lower responsiveness (slope) of PDE3B to its inhibitor in adipocytes from niacin treated mice, independent of insulin signaling.
Figure 3: PDE3B capacity of isolated adipocytes after lipolysis stimulation and inhibition. Glycerol concentration in ex vivo gonadal adipocyte medium after 2 h incubation with PDE3B inhibitor cilostazol. A) Responsiveness of basal and 8Br-cAMP stimulated lipolysis to PDE3B inhibition by different cilostazol concentrations. B) PDE3B capacity as glycerol release after PDE3B activation by 10^{-10} M insulin compared to PDE3B inhibition by 10^{-4} M cilostazol (Δ = 8Br-cAMP+Ins+Cilo - 8Br-cAMP+Ins) tested in ex vivo adipocytes from control and niacin treated mice. All values are means±SEM. N=10 mice per group, *p≤0.05, **p≤0.01 compared to niacin treatment, ###p≤0.001 compared to Cilo 10^{-6} M level.
Figure 4: Molecular mechanism of niacin action in adipocytes. Adrenergic stimulation of adipocytes will activate AC to produce cAMP. This rise in cAMP will both activate lipolysis via PKA & HSL and will activate PDE3B leading to a negative feedback loop degrading cAMP. Insulin will activate IRS1 and PKB, also leading to PDE3B activation, thereby inhibiting lipolysis. Prolonged niacin leads to down regulation of the β-adrenergic and insulin pathway, but concomitantly to a down-regulation of PDE3B. Whether this is a direct or indirect effect is unknown. The diminished PDE3B capacity dampens the negative feedback loop, resulting in an increased responsiveness to adrenergic and cAMP stimulation of lipolysis, as well as decreased responsiveness to insulin inhibition of lipolysis.
**Discussion**

The present paper shows that long term niacin induces insulin resistance in APOE*3-Leiden CETP female transgenic mice fed a Western type diet. The insulin resistance by niacin has been shown before in dyslipidemic patients and is confirmed in our humanized mouse model, both in vivo on glucose and glycerol metabolism and ex vivo in isolated adipocytes on lipolysis. The niacin induced insulin resistance was associated with a reduction in the expression of key insulin signaling genes (i.e. Insr and Irs1) in WAT. This indicates that niacin down-regulates the insulin signaling pathway at the transcriptional level which led to the reduced anti-lipolytic effect of insulin. In contrast, gene expression of all β-adrenergic receptors, as well as Hsl and Arrb1 were down-regulated in white adipose tissue, while an increased adrenergic lipolytic effect was observed. This might be due to desensitization after niacin-induced prolonged stress [10, 17-20]. Prolonged stress leads to increased adrenal gland size [21], also evident in our study. Decreased β-adrenoceptor gene expression combined with an increased β-adrenergic responsiveness suggests that the effects of niacin on β-adrenergic stimulation are due to post-receptor signaling mechanisms that enhance cAMP stimulation and thus lipolysis.

Interestingly, 8Br-cAMP, which acts at the post-receptor level, induced lipolysis to a higher extent in niacin treated mice, which strengthens the hypothesis that a post-receptor signaling mechanism is affected by niacin. The adipocyte lipolysis assays were not confounded by a difference in weight of the gWAT depots, nor in adipocyte mean cell volume (Table 1), which in itself might change responsiveness to reagents. It was shown by Oh et al. [10] that 24 h niacin infusion lowered the expression level of Pde3b in gWAT to a similar extent (-57%) as compared to our study (-53%). PDE3B regulates intracellular cAMP levels and is the key enzyme involved in the anti-lipolytic action of insulin. Moreover, by modulating cAMP levels PDE3B may interact with β-adrenergic signaling. Oh and co-workers suggested that the reduction in Pde3b may be an indirect effect of niacin, that serves as a counter-regulatory mechanism to increase the cellular cAMP/AMP ratio in order to maintain basal lipolytic rate.

In the current paper we show that the reduction of expression of Pde3b in adipose tissue by niacin is accompanied by a reduced anti-lipolytic effect of insulin. We further show by using the selective PDE3 inhibitor cilostazol in an adipocyte lipolysis assay that the PDE3B responsiveness in adipocytes isolated from long term niacin treated mice was diminished. We also showed that cilostazol increased 8Br-cAMP-stimulated lipolysis in a concentration dependent manner in adipocytes from control mice, whereas it did not have an effect on unstimulated cells.

These data suggest that in adipocytes PDE3B will only degrade cAMP when the lipolytic cascade is stimulated. These observations could explain the increased responsiveness to β-adrenergic or 8Br-cAMP stimulation in niacin treated adipocytes. In the unstimulated basal condition PDE3B was not activated, which corresponds to the unchanged adipocyte lipolysis rate and basal adipose tissue cAMP levels between control and niacin treated mice.
In line with this, plasma NEFA levels did not differ between the groups at the start of the \textit{ip} insulin tolerance test, indicating that \textit{in vivo} adipose lipolysis was also similar between the groups, despite decreased \textit{Pde3b} expression.

We propose a molecular mechanism for long term niacin treatment occurring in adipocytes (Fig. 4): Lipolysis is regulated by hormone sensitive lipase, which is controlled by cAMP levels. Production of cAMP can be stimulated via \(\beta\)-adrenergic pathways or inhibited by PDE3B which is activated by the insulin signaling cascade. PKA is activated by cAMP, which can activate PDE3B creating a negative feedback loop \([22-24]\) (Fig. 4). Long term niacin treatment leads to down-regulation of PDE3B (Fig. 4). This down-regulation of PDE3B is also accompanied by a down-regulation of the rest of the insulin signaling pathway, leading to reduced insulin responsiveness in the adipocyte. In addition, as the PDE3B responsiveness is smaller in adipocytes from niacin treated mice, stimulated cAMP levels in the niacin treated mice -after \(\beta\)-adrenergic stimulation- will be degraded to a lesser extent, leading to a net higher lipolysis.

In this study we have focused on adipocyte insulin response after long term niacin treatment. But the sequence of events leading up to adipocyte insulin resistance and decreased \textit{Pde3b} expression remain unclear, as does the possible role of adipocyte insulin resistance in the development of whole body insulin resistance. Moreover niacin has been shown to increase plasma adiponectin, an important insulin sensitizing hormone. The increase in adiponectin plasma levels after niacin treatment were shown to correlate to the decrease in insulin resistance \([25]\), indicating a possible compensatory insulin sensitizing effect. We however did not detect an increase of adiponectin gene expression in gWAT after niacin treatment (data not shown). Whether niacin acutely interferes with the insulin receptor cascade remains to be investigated, although PDE3B capacity was not affected in control adipocytes exposed to niacin for two hours (data not shown). Choi \textit{et al.} have shown a reduced phosphorylation of PKB/Akt in adipose tissue after 1.5h infusion of niacin \([26]\), which leads to a lower insulin response. Niacin thus seems to induce decreased adipocyte insulin responsiveness via more than one mechanism.

In conclusion, long term niacin treatment resulted in insulin resistance and increased \(\beta\)-adrenergic responsiveness which may in part be explained by a niacin induced down-regulation of PDE3B.

\textbf{Acknowledgements}

We thank S.J.M. van der Tuin for discussions on gene expression analysis. This work was supported by grants from the Center of Medical Systems Biology (CMSB), the Netherlands Consortium for Systems Biology (NCSB) established by The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NGI/NWO). MRB is supported by the Board of Directors of the Leiden University Medical Center (LUMC). PCNR is Established Investigator of the Netherlands Heart Foundation (NHS2009T038).
References


7. Soga T, Kamohara M et al. 2003; Molecular identification of nicotinic acid receptor. Biochemical and biophysical research communications 303:364.


