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Time-dependent regulation of muscle caveolin activation and insulin signalling in response to high-fat diet

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ABSTRACT

We studied the effect of high-fat diet on the expression and activation of the three caveolins in rat skeletal muscle and their association with the insulin signalling cascade. Initial response was characterized by increased signalling through Cav-1 and Cav-3 phosphorylation, suggesting that both participate in an initial acute response to the calorie surplus. Afterwards, Cav-1 signalling was slightly reduced, whereas Cav-3 remained active. Late chronic phase signalling through both proteins was impaired inducing a prediabetic state. Summarizing, caveolins seem to mediate a time-dependent regulation of insulin cascade in response to high-fat diet in muscle.

1. Introduction

Obesity is commonly attributed to an imbalance between food intake and energy expenditure resulting in the storing of energy excess as fat, primarily in adipose tissue and secondly in skeletal muscle, liver, pancreas and brain [1]. Different genes have been related to obesity development, such as the 3 major isoforms of caveolins, Cav-1, Cav-2 and Cav-3 (18–24 kDa), which are the main structural components of caveolae [2]. Cav-1 is most abundantly expressed in terminally differentiated cells, such as adipocytes, fibroblasts, epithelial and endothelial cells. Cav-2 is coexpressed with Cav-1, forming hetero-oligomers that require Cav-1 for proper membrane localization. Finally, Cav-3, whose function is similar to Cav-1, is considered as the muscle-specific member of the caveolin family, although it has also been found in astrocytes and chondrocytes [3,4]. Interestingly, Cav-1 is also expressed in skeletal muscle, where it interacts with Cav-3 in a specific manner [5].

Caveolae have been related to endocytosis of different molecules or particles, such as cholesterol and fatty acids [6], but they act also as scaffolding structures for the organization of a variety of signal transduction proteins [7]. Thus, there is considerable evidence of caveolin involvement in the modulation of insulin signalling and therefore in the regulation of the intermediary metabolism, which becomes altered in obesity, and associated with metabolic disorders such as insulin resistance and type 2 diabetes [7]. For instance, Cav-3 deficient mice develop late-onset obesity accompanied by insulin resistance in key peripheral tissues and abnormal lipid metabolism [8,9].

Therefore, it is clear that caveolins may play an important regulatory role in muscle insulin signalling. A better understanding of the insulin signalling deregulation that accompanies obesity is a prerequisite to improve therapeutic treatments. Thus, in this work we sought to investigate the effect of high-fat diet feeding in caveolin expression and activation in skeletal muscle and its relation with the deregulation of insulin signalling and the onset of a prediabetic state.

2. Materials and methods

2.1. Animals, diets and experimental design

Male Wistar rats (250–300 g), purchased from the Applied Pharmacobiology Center (CIFA, University of Navarra, Spain), were housed under controlled conditions of temperature (22 ± 2 °C), relative humidity (55 ± 10%) and light cycle (8 a.m. to 8 p.m). Fifty-six animals were assigned to two different dietary groups for 72 days. The control group ($n = 28$) had ad libitum access to standard

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laboratory pelleted diet (Harlan Iberica, Barcelona, Spain), whereas the second group ($n = 28$) was fed a fat-rich cafeteria diet [10]. Rats were sacrificed by decapitation, after overnight fasting, in four moments during the experimental period: 4, 12, 35 and 72 days. Each group was made up of eight control (*C*) and eight high-fat fed animals (HF), excepting the groups of day 72 ($n = 4$, each). After the sacrifice, trunk blood was collected and gastrocnemius skeletal muscle immediately isolated, frozen in liquid nitrogen, and stored at −80 °C. All the procedures were performed according to national guidelines and under care of the Animal Care and Use Committee at the University of Navarra.

2.2. Analysis of blood samples

Serum glucose was analyzed using a Cobas Mira autoanalyzer (Roche Diagnostics, Basel, Switzerland) by enzymatic routine procedures. Serum insulin levels were measured by ELISA (Mercodia AB, Uppsala, Sweden). HOMA index was calculated as fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5.

2.3. Reverse transcription (RT)-PCR

Total RNA was extracted from frozen skeletal muscle with Trizol (Invitrogen, Carlsbad, CA, USA), purified by DNase treatment (DNA-free™ kit, Applied Biosystems, Austin, TX, USA) and used as a template to generate first strand cDNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed as described by the provider (Applied Biosystems, Austin, TX, USA) using an ABI-PRISM 7300 HT Sequence Detection System and Taqman probes for rat Cav-1, Cav-2, Cav-3, GLUT-4, IR, IRS-1 and IRS-2. GAPDH was used as internal control for efficiency of RT-PCR and subsequent normalization. The results were calculated by the $2^{-\Delta\Delta Ct}$ method [11].

2.4. Western blot analyses

For Western blot analysis, skeletal muscle samples were homogenized with lysis buffer. Ten to fifteen micrograms protein were electrophoretically separated and incubated with specific primary antibodies at the suitable dilution and following a standard protocol [12]. The blots were stripped by immersion in Red-Blot Plus solution 10X for 10 min at room temperature (Millipore, Billerica, MA, USA) and reprobed with different antibodies, using the same procedure as described above. Cav-1 (1/50 000), Cav-2 (1/20 000) and Cav-3 (1/10 000) antibodies were from Santa Cruz Biotechnology Inc., CA, USA; Insulin Receptor (1/10 000) and Phosphotyrosine (1/10 000), from Cell Signalling Technology. The secondary antibodies were Anti-Mouse (1/10 000), from Amersham Biosciences-GE Healthcare, WI, USA, Anti-Rabbit (1/10 000), from Sigma Aldrich, St. Louis, MO, USA, and Anti-Goat (1/10 000), from Santa Cruz Biotechnology Inc.

2.5. Inmunoprecipitation

For immunoprecipitation, samples containing 500 μg of total protein were incubated with antibodies against Cav-1 (1/100 v/w), Cav-3 (1/100 v/w) and IR (1/50 v/v) following the supplier's protocols (Santa Cruz Biotechnology Inc. and Cell Signalling Technology), before being analyzed by Western blot with the corresponding antibodies, as described above, and quantified by densitometry.

2.6. Data analysis

The results were expressed as the mean ± S.E.M. Data were compared using Mann–Whitney *U* test, while correlation analyses were performed using Spearman's *rho*. All analyses were performed using SPSS version 15.0 for Windows.

3. Results

3.1. Body weight and blood determinations

Rats consuming the high-fat diet (HF) gained significantly more weight ($P < 0.05$) than those receiving the control diet, increasing body fat percentage and decreasing relative muscle weight with respect to total body weight since day 12 (Fig. 1). They also developed hyperglycemia, hiperinsulinemia and insulin resistance at the end of the experimental period (Fig. 1).

3.2. mRNA and protein levels of caveolins

Cav-3 mRNA, measured by RT-PCR, resulted significantly increased in HF-fed rats from day 12 as compared to the control group (Fig. 2). Cav-1 and Cav-2 show a similar expression pattern than Cav-3, although the differences were not significant (Fig. 2).

Caveolin protein contents in skeletal muscle, quantified by Western blot analysis, were not affected by the diet (Fig. 3A). However, Cav-1 and Cav-3 show a positive and Cav-2 an inverse correlation to body weight (Fig. 3B).

3.3. Protein phosphorylation of Cav-1, Cav-3 and IR

Phosphorylation levels of IR, Cav-1 and Cav-3 were increased in HF-fed rats at day 4, as a sort of initial response (Fig. 4), more evident for IR ($P < 0.05$) and Cav-3 ($P = 0.053$). However, from day 12 ahead, IR phosphorylation level in HF animals was the same as in control fed rats (Fig. 4), indicating that there is no response of IR to the increased insulin circulating level.

3.4. mRNA expression of insulin pathway proteins

Neither IR nor GLUT4 mRNAs were modified by the diet (Fig. 5). IRS-1 increased its expression in HF animals at day 4, as an early response to the diet, but from day 12 ahead, it gradually decreased ($P < 0.05$ at day 72). On the other hand, IRS-2 showed an inverse expression pattern to IRS-1, being inhibited up to day 35 but significantly increased at day 72 (Fig. 5).

4. Discussion

After 10 weeks of high-fat cafeteria diet, rats developed a prediabetic state characterized by hyperinsulinemia and insulin resistance, as previously reported [13]. Skeletal muscle is the major organ responsible for glucose uptake under insulin-stimulated conditions [14], and is generally considered the most important site for insulin resistance [15]. An impairment of the initial steps of insulin signalling transduction pathways could contribute to the defect in insulin-stimulated glucose uptake in skeletal muscle, thus resulting in insulin resistance. In fact, two mechanisms have been described in relation to lipid-induced muscle insulin resistance: acute free fatty acid elevation, and prolonged muscle lipid accumulation [16].

In this study, increased insulin resistance in animals fed a fat-rich diet is evidenced by reduced insulin receptor (IR) phosphorylation since day 12 and IRS-1 mRNA at day 72, confirming data from other groups [17]. Cav-1 is also downregulated since day 12, suggesting that this protein may be involved in the malfunction of the classical insulin pathway that could result in insulin resistance [18].

It is known that caveolin subtypes 1 and 3 play an important role in maintaining insulin signalling and cellular energy metabo-