Serum from Calorie-Restricted Rats Activates Vascular Cell eNOS through Enhanced Insulin Signaling Mediated by Adiponectin

Fernanda M. Cerqueira¹, Laura I. Brandizzi², Fernanda M. Cunha¹,³, Francisco R. M. Laurindo², Alicia J. Kowaltowski¹*

¹ Instituto de Química, Departamento de Bioquímica, Universidade de São Paulo, São Paulo, São Paulo, Brazil, ² Instituto do Coração, Faculdade de Medicina, Universidade de São Paulo, São Paulo, São Paulo, Brazil, ³ Escola de Artes, Ciências e Humanidades, Universidade de São Paulo, São Paulo, São Paulo, Brazil

Abstract

eNOS activation resulting in mitochondrial biogenesis is believed to play a central role in life span extension promoted by calorie restriction (CR). We investigated the mechanism of this activation by treating vascular cells with serum from CR rats and found increased Akt and eNOS phosphorylation, in addition to enhanced nitrite release. Inhibiting Akt phosphorylation or immunoprecipitating adiponectin (found in high quantities in CR serum) completely prevented the increment in nitrite release and eNOS activation. Overall, we demonstrate that adiponectin in the serum from CR animals increases NO signaling by activating the insulin pathway. These results suggest this hormone may be a determinant regulator of the beneficial effects of CR.

Results

CR decreases serum glucose and insulin; increases adiponectin levels

After 26 weeks of CR, the average body weight of rats was lower than control AL rats, an effect accompanied by lower visceral fat deposits, serum glucose, insulin, and increased adiponectin levels (Table 1), alterations similar to those observed in most literature CR studies [28].

CR serum increases NO\(^{\text{•}}\) production

VSMC cells incubated in media in which standard serum was substituted for serum collected from CR rats presented a time-dependent increase in NO\(^{\text{•}}\), indicative of higher levels of NO\(^{\text{•}}\) production compared to cells maintained in media containing serum from animals fed AL (Fig. 1A). This result shows that acute treatment with serum from CR animals is sufficient to increase VSMC NO\(^{\text{•}}\) production, and suggests CR serum contains regulatory signals leading to this effect.

Introduction

Caloric restriction (CR) extends lifespans of model organisms ranging from yeast to mammals [1–4], and many groups have focused on understanding how this dietary intervention acts mechanistically. In 2005, Nisoli and collaborators [5] elegantly demonstrated that dietary restriction induced the activation of endothelial nitric oxide synthase (eNOS) and lead to enhanced mitochondrial biogenesis and increased oxygen consumption. Indeed, the effects of the diet were largely absent in eNOS deficient animals [5]. Further studies have found links between mitochondrial activity and CR. Fungal CR models present decreases in blood glucose [31]. We investigate here if changes in serological profiles in CR animals are sufficient to uncover the signaling pathways involved.
We sought to determine the source of this augmented NO\(^*\) production by measuring the activities of eNOS in cells which had been cultured in AL media and were then switched to media containing serum from CR animals. Under these conditions, the quantity of total eNOS increased significantly after 24 h (by 203\(\pm\)8\%, \(p<0.05\)). Furthermore, active, phosphorylated, eNOS increased (Fig. 1B shows a representative blot of the time-dependent effect of incubation in CR serum, while Fig. 1C quantifies relative phosphorylated band intensity after 24 h in AL or CR sera). Overall, these results indicate that eNOS expression and activation is promoted by serological changes induced by CR.

CR serum increases insulin signaling

We have previously shown that Akt and eNOS are activated in insulin-sensitive tissues of CR animals [13]. We sought to measure the activity of this pathway in VSMC cells cultured in the presence of CR serum (Fig. 2) and found that the active, phosphorylated, form of Akt increased in a time-dependent manner in CR media, from undetectable levels in AL serum (Fig. 2A, upper panels). Indeed, after 24 h in CR serum, a highly significant change in p-Akt levels was detected relative to AL serum (Fig. 2B).

Among other pathways controlling Akt, this protein is sensitive to insulin signaling. Although insulin levels in CR serum are decreased relative to AL (Table 1), we measured the activation of insulin receptors (IR) from VSMC grown 24 h in AL and CR media. The receptors were immunoprecipitated and probed with anti-phospho-Tyr antibodies. CR serum significantly enhanced the total amount of IR by 194\(\pm\)8%, \(p<0.05\), and lead to a strong increment in receptor phosphorylation (Figs. 2A and 2B), indicating that it contains components other than insulin capable of acutely activating the insulin pathway.

CR serum-induced NO\(^*\) release is dependent on Akt

In order to investigate if enhanced NO\(^*\) release from VSMC cells was dependent on the activation of the insulin pathway, we inhibited Akt activity with 1 \(\mu\)M naphthyridinone 17 (NTD). This concentration of NTD completely prevented the accumulation of NO\(^*\) promoted by CR serum, but did not affect the release in cells grown in AL serum (Fig. 3A). Furthermore, NTD completely eliminated the detection of phospho-eNOS and decreased total eNOS band intensity (Fig. 3B). This is consistent with the finding that Akt activity is important for eNOS phosphorylation [21].

Adiponectin mediates the activation of the insulin pathway and NO\(^*\) release induced by CR serum

The activation of the insulin pathway in cells acutely treated with CR serum is surprising since insulin levels are lower (Table 1). However, adiponectin levels are increased in CR, and this hormone is an activator of the insulin pathway [32,33]. To address the role of adiponectin in the CR serum effect on NO\(^*\) release, we removed it through immunoprecipitation. The procedure was highly effective (Fig. 4A). Using immunoprecipitated sera, we noted that the phosphorylation of insulin receptors promoted by CR serum was eliminated (Fig. 4B), while no effect was seen in AL serum. Immunoprecipitation of adiponectin also totally reversed the effect of CR serum on eNOS phosphorylation (Fig. 4C) and on NO\(^*\) release (Fig. 4D). Overall, these results indicate that enhanced NO\(^*\) release promoted by CR serum in vascular cells is a consequence of high adiponectin levels.

Discussion

Mitochondrial mass and function decrease during aging [34–36] in a manner prevented by CR, which promotes enhanced NO\(^*\) signaling associated with mitochondrial biogenesis [5,6,11,13]. Thus, NO\(^*\) signaling seems to be central toward the beneficial effects of CR in aging, although the mechanisms through which CR affects this pathway have not been directly approached to date. We addressed this point by treating VSMC, prone to respond to physiological stimuli that affect NO\(^*\) release [37,38], with serum collected from CR animals. This protocol has the advantage of separating long-term dietary effects from acute effects on vascular cells, specifically addressing the question if hormonal changes in CR are sufficient to activate NO\(^*\) signaling.

We observed a time-dependent increment in NO\(^{\text{2-}}\) released into the culture medium, indicative of enhanced NO\(^*\) production, as well as increments in eNOS quantity and phosphorylation (Fig. 1), a result in line with previous data showing that CR

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Table 1. Effects of CR and AL diets.

|                | CR   | AL   | P value |
|----------------|------|------|---------|
| Body weight (g) | 481.5±82.9 | 675.7±93.1 | <0.0001 |
| Visceral fat (g) | 23.9±9.8 | 31.2±7.9 | 0.0009  |
| Serum glucose (mg dL\(^{-1}\)) | 85.8±3.7 | 115.1±6.6 | 0.0008  |
| Serum insulin (ng mL\(^{-1}\)) | 0.58±0.29 | 1.98±0.85 | <0.0001 |
| Serum adiponectin (relative to AL) | 3.0±0.7 | 1 | <0.0001 |

Measurements were conducted as described in Materials and Methods. doi:10.1371/journal.pone.0031155.t001
induced the expression of eNOS through Akt [5, 13, 17, 19, 20]. Indeed, Akt phosphorylation was strongly enhanced by CR serum (Fig. 2) and NTD (a selective Akt inhibitor when used at low micromolar doses [39]) inhibited eNOS phosphorylation (Fig. 3).

The insulin receptor, an upstream regulator of Akt activity and eNOS activation [40], was also activated by CR serum (Fig. 2). Insulin signaling is well known to activate NO signaling, and Akt physically interacts with eNOS in response to insulin [41]. However, insulin is found at decreased levels in CR serum while adiponectin, an activator of peripheral insulin signaling [32, 33], is increased (Table 1, [42, 43]). Furthermore, adiponectin was previously reported to activate eNOS through Akt [44, 45].

Accordingly, we sought to determine if adiponectin in CR serum could activate NO signaling. We immunoprecipitated adiponectin from both AL and CR sera (Fig. 4A), and found that, while this did not alter the release of NO2+ promoted by AL serum, it completely abrogated the increased release specific to CR serum (Fig. 4D). In addition, increased activation of the insulin pathway and eNOS were absent upon removal of adiponectin (Figs. 4B and C). Together, these results demonstrate that adiponectin is the key regulator of enhanced NO signaling in vascular cells stimulated with CR serum.

It should be noted that VSMCs present different signaling receptors and pathways than endothelial cells, which could thus present different responses to CR sera. However, previous results demonstrate that adiponectin stimulates NO release from endothelial cells [46], supporting the idea that this cytokine is probably a key signaling molecule in CR-induced NO signaling. Interestingly, it seems that eNOS-derived NO can also have a determinant role in regulating the production of adiponectin by adipocytes [47].

Overall, our results point to adiponectin as a key serological factor involved in acute cellular responses altered by CR, and suggest that this hormone may be a central regulator of
mitochondrial biogenesis and other processes involving NO signaling.

Materials and Methods

Animals and serum collection

All experiments were conducted in agreement with National Institutes of Health guidelines for humane treatment of animals and were approved (unnumbered) the local Animal Care and Use Committee (Comissão de Ética em Cuidados e Uso Animal), Male, 8-week-old Sprague-Dawley rats were separated into 2 groups: AL, fed ad libitum with an AIN-93-M diet prepared by Rhoster (Campinas, SP, Brazil) and CR, fed at levels 60% of AL ingested. Rats were fasted for 12 hours and used for glucose analysis (Accu-Check Performa Glucose Analyzer, São Paulo, SP, Brazil). For insulin and adiponectin determinations, blood samples were centrifuged at 2,000 g for 15 min and the supernatant was stored at −20°C. Insulin was measured using a Lincos Research ELISA kit (St. Charles, MO, USA). Adiponectin was detected by Western Blot.

Serum analysis

Insulin, glucose, triglycerides, HDL, total cholesterol and adiponectin levels from AL or CR sera were evaluated (Table 1). Peripheral blood was collected from the tail of 40-week-old animals fasted for 12 hours and used for glucose analysis (Accu-Check Performa Glucose Analyzer, São Paulo, SP, Brazil). For insulin and adiponectin determinations, blood samples were collected at 1000 g for 15 min and the supernatant was stored at −20°C. Insulin was measured using a Lincos Research ELISA kit (St. Charles, MO, USA). Adiponectin was detected by Western Blot.

Cell cultures

Rat vascular smooth muscle cells (VSMC) were purchased from ATCC (CRL-2797) and cultured in 25 mM glucose DMEM supplemented with 18 mM sodium bicarbonate, 4 mM glutamine, 0.3 mM glutamic acid, 100 μg/mL streptomycin, 100 U/mL penicillin and 10% v/v fetal bovine serum, at 37°C and 5% CO2. Cells were passaged every 3 days. After the 8th passage, cells were incu-802bated with an AIN-93-M diet prepared by Rhoster (Campinas, SP, Brazil) and CR, fed at levels 60% of AL ingested amounts a diet supplemented with micronutrients to reach the vitamin and mineral levels consumed by AL animals [48]. Food was offered daily at 6 pm and feedings were adjusted weekly by weight, based on AL food consumption. The intervention resulted in known alterations associated with CR including lower body weight and improved insulin sensitivity [49]. The animals were lodged 3 per cage and given water ad libitum. At 34 weeks (26 weeks on the diet), rats were sacrificed after 12 hours fasting and the serum was obtained as described in [50], allowed to clot for 20–30 min at 25°C and centrifuged for 20 min at 300 g. The supernatant was collected and stored (−20°C), thawed and heat-inactivated at 56°C for 30 min prior to use.

Data analysis and statistics

Data shown are representative blots or averages ± SEM of at least three identical repetitions. Data were analyzed using GraphPad Prism and compared using t-tests (for data pairs) or two-tailed ANOVA followed by Tukey tests (multiple comparisons).

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Author Contributions
Conceived and designed the experiments: F.M. Cerqueira F.M. Cunha FRML AJK. Performed the experiments: F.M. Cerqueira LIB F.M. Cunha. Analyzed the data: F.M. Cerqueira F.M. Cunha. Contributed reagents/materials/analysis tools: FRML AJK. Wrote the paper: F.M. Cerqueira AJK.

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