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Sulforaphane effects on oxidative stress parameters in culture of adult cardiomyocytes

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ABSTRACT

The aim of this study was to analyse the effect of sulforaphane (SFN) in cultures of adult cardiomyocytes, evaluating oxidative stress at different times. Cells were isolated, cultured, and divided into 4 groups: Control, SFN (5μM), H_2O_2 (5μM), and SFN + H_2O_2 (5μM both), and subdivided into groups undergoing 1 or 24 h of SFN incubation. After 1 h of incubation, reactive oxygen species production was 40% lower in the SFN group than the Control, and lipid peroxidation was 63% higher in the H_2O_2 group than the Control, and it was reduced in both of the SFN groups. The SOD activity was 59% higher in groups incubated for 24 h than in those incubated for 1 h. Protein expression of SOD-1 and SOD-2 was higher in the 24-h groups compared to the 1-h groups (55% and 24%, respectively). The Nrf2 protein expression in the 1-h groups was 17% higher than in the 24-h groups, and the SFN + H_2O_2 group had 40% more Nrf2 than the Control in the 1-h groups. Unlike Nrf2, the PGC-1α expression was 69% higher in the 24-h groups in relation to the 1-h groups. Regarding the 24-h groups, the SFN and SFN + H_2O_2 groups were higher than the Control (32% and 33%, respectively), and the SFN + H_2O_2 group was increased (21%) compared to H_2O_2. SFN had a protective action against oxidative damage, but had no effect on the antioxidant enzymes analyzed. The different responses in the expression of Nrf2 and PGC-1α in relation to the incubation times, draws attention to the importance of establishing a timeline of the action of SFN, since there appears to be a temporal difference in its mechanism in adult cardiomyocytes.

1. Introduction

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables, such as broccoli, cabbage, radish, and cauliflower [1,2]. Data from the literature show that this compound has antioxidant, anti-inflammatory, and anti-tumour properties [3]. A human-based study showed that the consumption of broccoli sprouts for 1 week decreased markers of oxidative stress and improved cholesterol metabolism [4]. The study by Angeloni et al. [5] showed that, at a concentration of 5μM, SFN protected cardiac cells from neonatal rats from oxidative damage and induced a series of antioxidant enzymes.

However, the effect of SFN is different depending on the dose administered or ingested. Therefore, it can be stated that SFN is used in both antioxidant and anti-tumour therapies, since it has an adaptive response to oxidative stress at low concentrations and a pro-apoptotic action, which is an important anticancer property, at high levels [2]. This biphasic behaviour is called hormesis [6].

Classically, the action of sulforaphane in the induction of antioxidant defenses is related to activation of the transcription factor Nrf2 (nuclear erythroid type 2 factor) [5]. However, the action of SFN on the activation of another transcription factor, PGC-1α (peroxisome proliferator-activated receptor co-activator-1 alpha), has been increasingly explored. Negrette-Guzmán et al. [7] described the induction of mitochondrial biogenesis through the activation of PGC-1α as one of the effects of SFN. In another study, the activity and expression of PGC-1α were decreased in diabetic cardiomyopathy, and SFN was able to restore these levels, thus improving mitochondrial function and oxidative capacity [8]. These data suggest that the induction of antioxidant factors by SFN is not only modulated by Nrf2, as seen in most studies, but may also be via PGC-1α, a fact that has not been well explored in the literature.

Based on the above-mentioned data and on the fact that a previous study demonstrated the cytoprotective effect of SFN in cardiomyoblasts (H9c2 cells), without altering the expression of Nrf2 but increasing the

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expression of PGC-1α [9], it is important to study how this effect occurs in isolated adult cardiomyocytes, since the antioxidant response in these two cell types may be different. In addition, data from the literature show that the activation of Nrf2 and PGC-1α is often not continuous and suffers variations according to the time at which they are induced [10–12]. Thus, the analysis of these transcription factors in the culture of isolated adult cardiomyocytes may help to better elucidate SFN activity in these pathways.

Therefore, the aim of this study was to explore the cardioprotective effect of sulforaphane in adult cardiomyocytes, through the evaluation of oxidative stress parameters and antioxidant defenses, at different times and in non-oxidizing and oxidizing conditions.

2. Materials and methods

2.1. Ethical approval

All of the experimental procedures developed in the present study were performed taking into consideration the welfare of all animals. This project was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul (CEUA/UFRGS), with process number 28583.

2.2. Animals and experimental groups

Adult male Wistar rats weighing between 200 and 250 g, from the Animal Reproduction and Experimentation Center of UFRGS (CREAL-UFRGS), were kept in plastic cages, under standard conditions, with a light/dark cycle of 12 h, a temperature of 21 °C, and controlled humidity (55%), along with free access to water and food.

Four experimental groups of adult cardiomyocytes isolated in culture were established: [1] **Control group** (cells in culture medium 199, containing 0.5% FBS); [2] **SFN group** (cells in culture medium 199, containing 0.5% FBS and 5μM sulforaphane); [3] **H_2O_2 group** (cells in culture medium 199, containing 0.5% FBS, and incubated with 5μM H_2O_2 for 15 min); [4] **SFN + H_2O_2 group** (cells in culture medium 199, containing 0.5% FBS, 5μM sulforaphane, and 5μM H_2O_2 for 15 min). The groups were further subdivided into the different SFN incubation times: 1 and 24 h.

2.3. Isolation and culture of adult rat cardiomyocytes

The animals received a heparin intraperitoneal (i.p.) injection and were then euthanized with the i.p. administration of thiopental (120 mg/kg) mixed with lidocaine (10 mg/mL), to induce anaesthesia overload, for withdrawal of the heart. Procedures for the isolation and culture of adult cardiomyocytes were performed through the enzymatic digestion of cardiac fibrous tissue, as described by Lou et al. [13]. The heart was removed from the animal and immediately placed into a modified Langendorff's system, through cannulation of the aorta. First, the heart was perfused with a modified Krebs (37 °C) heated solution for 4–5 minutes [14]. After that, heart was perfused with Krebs solution containing Type II collagenase for 30 min (constant flow of 4 mL/min at 37 °C). After perfusion, tissue was gently separated by surgical clamps and the solution containing isolated cells was filtered through a 200 μm filter. The pellet formed was resuspended in culture medium 199 containing FBS 5%, antibiotic 0.1% (gentamycin) and antifungal 0.2% (amphotericin B). Cardiomyocytes (10^4 cells/cm^2) were then plated on culture plates (previously coated with laminin 20 μg/ml - Sigma-Aldrich) and kept in a 5% CO_2 incubator at 37 °C. Then, cells were treated with H_2O_2 (5μM) for 15 min and/or SFN (5μM) for 1 or 24 h.

2.4. Cell viability

The cell viability of adult cardiomyocytes was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) [15] technique adapted for this model culture. The absorbance was quantified at 570 nm (650 nm correction wavelength) in a Zenyth 200rt reader (Biochrom Inc, UK) [16]. The data presented were expressed as a percentage of the control, as described by Bryan et al. [17]. This technique was used to test the concentration and the experimental incubation time with H_2O_2. The H_2O_2 concentrations tested were 5 and 10 μM (diluted in the culture medium) at the incubation times of 10 and 15 min (prior the incubation with SFN). To assess the effect of SFN concentrations on cell viability, the following concentrations were tested: 5 and 10 μM [5]. The cells were incubated with SFN for 24 h and then the cell viability test was performed.

2.5. Sample preparation and protein concentration measurements

After the respective cell incubation times, culture medium was gently removed from the wells and 10x Cell Lysis Buffer solution (Cell Signaling Technology, USA) was added to each one, following the manufacturer's instructions. The cells were scraped and collected from the plate. The samples were then sonicated (sonicator bath Ultrasonic Cleaner) with 4 pulses of 30 s, and centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatants were collected for biochemical analysis. Protein levels were measured using Lowry's method [18], and bovine serum albumin was used as a standard.

2.6. Reactive oxygen species levels

Determination of the levels of total reactive oxygen species (ROS) was performed by fluorescence emission of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, USA), a technique that was first described by Lebel et al. in 1992 [19]. The reaction was measured in a fluorescent spectrophotometer, in which the samples were excited at 488 nm and the fluorescence emission was collected at 525 nm. The results were expressed in nmol of formed DCF/mg protein.

2.7. Lipid peroxidation levels

Lipid peroxidation was evaluated in a beta-counter (LKB Rack Beta Liquid Scintillation Spectrometer-1215; LKB Produkter AB, Brommma, Sweden). The samples (0.5 mg/mL) were placed in low potassium vials, in a reaction medium consisting of phosphate buffer (30 mmol/L) and KCl (120 mmol/L) (pH = 7.4). First, the spontaneous emission (initial) of the samples was measured; then, the chemiluminescence reaction was initiated by the addition of tert-butyl hydroperoxide (t-BOOH) and followed until it reached the peak (maximum emission). The levels of lipid damage were determined by the difference between the maximum and initial emission values. Results were expressed as cps/mg protein [20].

2.8. Antioxidant enzyme activities

The SOD activity was based on the inhibition of pyrogallol superoxide radical reaction in alkaline medium (pH = 10) with pyrogallol as described by Marklund in 1985 [21]. The reaction was measured in a spectrophotometer at 420 nm, and enzyme activity was expressed as U/mg protein.

CAT activity was quantified based on the fact that its activity is directly proportional to the H_2O_2 decomposition rate. The reaction was measured in a spectrophotometer at 240 nm and the results were expressed in pmol/min/mg protein [22].

Finally, GST activity was quantified by the reaction of GST with chlorine dinitro-benzene (CDNB), which generates a compound that is conjugated with GSH, forming a dinitrophenyl glutathione coloured product. The reaction was measured in a spectrophotometer at 340 nm, and the results were expressed as pmol/min/mg protein [23].