The Adipokine Chemerin Induces Apoptosis in Cardiomyocytes

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Key Words
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Abstract

Background: The adipokine chemerin has been associated with cardiovascular disease. We investigated the effects of chemerin on viability and intracellular signalling in murine cardiomyocytes, and the effects of insulin and TNF-α on cardiomyocyte chemerin production.

Methods: Hoechst dye vital staining and cell cycle analysis were used to analyse the viability of murine cardiac cells in culture. Western blot was used to explore the phosphorylation of AKT and caspase-9 activity in neonatal rat cardiomyocytes and HL-1 cells. Finally, RT-qPCR, ELISA and western blot were performed to examine chemerin and CMKLR1 expression after insulin and TNF-α treatment in cardiac cells.

Results: Chemerin treatment increased apoptosis, reduced phosphorylation of AKT at Thr308 and increased caspase-9 activity in murine cardiomyocytes. Insulin treatment lowered chemerin and CMKLR1 mRNA and protein levels, and the amount of chemerin in the cell media, while TNF-α treatment increased chemerin mRNA and protein levels but decreased expression of the CMKLR1 gene.

Conclusion: Chemerin induces apoptosis, reduces AKT phosphorylation and increases the cleavage of caspase-9 in murine cardiomyocytes. The expression of chemerin is regulated by important metabolic (insulin) and inflammatory (TNF-α) mediators at cardiac level. Our results suggest that chemerin could play a role in the physiopathology of cardiac diseases.

Introduction

Obesity, considered to be a chronic state of systemic inflammation, is associated with the development of type 2 diabetes mellitus (T2DM) and several cardiovascular diseases,
including hypertension and atherosclerosis [1, 2]. Adipose tissue produces and releases a large number of adipokines, proteins with autocrine, paracrine and endocrine activity [3]. In obesity, alterations of adipocyte endocrine function include decreased secretion of anti-inflammatory adipokines such as adiponectin, and increased release of pro-inflammatory adipokines such as TNF-α, IL-6 and resistin [4]. Thus, the metabolic alterations observed in obesity are associated with a likely imbalance of pro- and anti-inflammatory cytokines [4]. Recent work has in fact shown that balanced adipokine production by adipose tissue is critical for systemic homeostasis, and that abnormal adipokine production is related to cardiovascular physiopathology [5].

The adipokine chemerin, also known as TIG2 (tazarotene-induced gene 2) or RARRES2 (retinoic acid receptor responder protein 2), is an 18 kDa protein produced mainly in the liver and in adipose tissue but also in many other tissues, including those of the cardiovascular system [6–8]. It is secreted as an inactive form (prochemerin) that is subsequently transformed into an active protein by protease cleavage of the C-terminal domain [9]. Human studies have demonstrated that chemerin is directly linked to different human pathophysiological states that include obesity, inflammation and insulin resistance [10] and support a connection between chemerin, inflammation, obesity and metabolic syndrome, a group of metabolic disorders that increase the risk of diabetes and several cardiovascular diseases. Therefore, the experimental and clinical investigations support a role for chemerin in normal physiology and in the pathophysiology of inflammation and metabolic disorders [11].

Although chemerin was initially described as a pro-inflammatory chemotactic peptide that induces immune cell migration in plasma [6], more recent studies have uncovered multiple additional roles in a wide range of inflammatory and metabolic processes, and chemerin levels appear to be modulated by both inflammatory and metabolic factors [11–13]. TNF-α treatment increases chemerin mRNA levels in 3T3-L1 adipocytes and mouse serum [12], but down-regulates chemerin gene expression in renal proximal tubular epithelial cells (in spite of which there is a simultaneous increase in bioactive chemerin, suggesting a role of TNF-α in the conversion of prochemerin to chemerin) [14]. IL1β, another pro-inflammatory cytokine that is also involved in insulin resistance, increases both chemerin mRNA and protein levels in 3T3-L1 adipocytes [13, 15].

Chemerin has been identified as the ligand of the G-protein-coupled receptor CMKLR1/ChemR23 (Chemokine like receptor 1), and more recently has also been found to bind to two other receptors, GPR1 (G-protein-coupled receptor 1) and CCR2 (C-C chemokine receptor-like protein 2) [16, 17]. The binding of chemerin to CMKLR1 has been shown to involve G proteins, intracellular calcium release and the regulation of MAPK ERK1/2, NF-kB and phosphatidylinositol 3-kinase/AKT (PI3K/AKT) signalling [10]. The kinase AKT is an important mediator and regulator of multiple signalling pathways that include metabolism, proliferation, differentiation, and apoptosis [18–20]. Several studies have shown that chemerin can affect AKT phosphorylation in different tissues and cells provoking changes in cell metabolism [21–26].

Chemerin has indeed been proposed as a predictive marker of cardiovascular risk [27, 28], and previous works have demonstrated that circulating chemerin concentrations correlate with several cardiometabolic parameters and with the severity of coronary artery disease [29–31]. Moreover, chemerin and CMKLR1 genes are both expressed by cells of the cardiovascular system, in which their levels have been shown to be positively correlated with the severity of cardiac pathologies such as atherosclerosis [32].

In spite of the evidence of its relationship with cardiovascular disease, chemerin has not hitherto been studied intensively in cardiomyocytes. In this study we investigated the effects of chemerin on cardiomyocyte viability and the effects of two central modulators of cardiovascular physiopathology (the pro-inflammatory molecule TNF-α and the cardioprotector insulin) on the cardiac production of this adipokine.
Materials and Methods

Ethics
All animals were maintained and euthanized following protocols approved by the Animal Care Committee of the University of Santiago de Compostela in accordance with European Union Directive 2010/63.

Cell cultures
Cardiomyocytes from neonatal rat hearts and adult mouse atrial HL-1 cardiomyocytes (a gift of Dr. W. C. Claycomb of Louisiana State University Medical Center, New Orleans, Louisiana), were all cultured as described previously [33]. Serum-deprived HL-1 cells and neonatal rat cardiomyocytes were treated for 6-48 hours with recombinant mouse TNFα (0.1–20 ng/ml) (Sigma Aldrich, USA), recombinant human insulin (0.1-100 nM) (Sigma Aldrich, USA) or recombinant mouse chemerin (0.1-100 nM) (R&D Systems, USA).

Hoechst dye vital staining
HL-1 cells and neonatal rat cardiomyocytes were seeded in 24-well plates, deprived of serum for 12 hours, and treated for 12-48 hours with 0.1 to 100 nM recombinant mouse chemerin. When the treatment finished the cells were incubated for 45 min at 37 °C in the culture medium with Hoechst 33258 dye (Sigma Aldrich, USA) in HEPES (pH 7.8) at a final concentration of 20 mM, and the cells were examined by fluorescence microscopy.

Cell-cycle analysis
HL-1 cells or neonatal rat cardiomyocytes were seeded in 6-well plates, deprived of serum for 12 hours, and treated for 24 hours with 100 nM recombinant mouse chemerin. When the treatment finished, 4% paraformaldehyde was added, followed, after 5 minutes, by 70% ethanol. After overnight incubation at -20°C, cell pellets were obtained and incubated for 30 minutes in a solution containing 1 mg/mL RNase and 5 μg/mL propidium iodide, after which flow cytometry was performed in a FACSARia apparatus (Becton & Dickinson, USA) using the FACSdiva software. Apoptosis was measured as the percentage of DNA in the hypodiploid (sub-G$_0$/G$_1$) peak.

RT-qPCR
RT-qPCR for chemerin and its receptor, CMKLR1, was performed on RNA extracted with the RNeasy Total RNA Extraction Kit (Qiagen, DE) using Master Mix and specific primers provided by Qiagen (rat Rarres2, 131 bp, Cat. No. PPR48247A, reference position 102, GenBank NM_001013427.1; rat CMKLR1, 152 bp, Cat. No. PPR06609, reference position 112, GenBank NM_022218.2; rat Gapdh, 172 bp, Cat. No. PPR06557A, reference position 363, GenBank NM_017008.3; human RARRES2, 96 bp, Cat. No. PPH02377A, reference position 515, GenBank NM_002889.3; human CMKLR1, 165 bp, Cat. No. PPH02349A, reference position 544, GenBank NM_004072.2; human GAPDH, 175 bp, Cat. No. PPH00150E, reference position 1287, GenBank NM_002046.3). Results were analysed using MxPro v4 software (Agilent, USA).

Western blotting
Cultured cardiomyocytes (50,000 cells/cm$^2$) or heart tissues were lysed and subjected to SDS-PAGE/Western blotting as previously described [34], using antibodies against chemerin and CMKLR1 (Santa Cruz Biotechnology, USA) at 1:200 dilution, and against ERK1/2 and phospho-ERK1/2 (Thermo Fisher Scientific, USA), AKT, Thr-308–phosphorylated AKT and Ser-473–phosphorylated AKT (Cell Signaling Technology, USA), AMPK and phospho-AMPK (Cell Signaling Technology, USA), P38 and phospho-P38 (Abcam, UK), Caspase-9 (Cell Signaling Technology, USA) and Gapdh (Sigma Aldrich, USA), all at 1:1000 dilution.

ELISA
The levels of chemerin secreted by neonatal rat cardiomyocytes were determined in the culture medium in 6-well plates in which cells had been cultured for 3 days at a density of 4.5x10$^4$ cells/well and after treatment with 20 ng/ml TNFα or 100 nM insulin for 48 hours by ELISA (Abbexa, UK), according to the manufacturer’s instructions.
Statistical analyses

All experimental data were obtained from at least 3 independent experiments, and are expressed as means ± SEMs. Comparisons between groups were performed using Student’s t tests for Gaussian data and Mann-Whitney U, Kruskal-Wallis, and Wilcoxon signed rank tests for non-Gaussian data. Statistical significance was accepted at the P < 0.05 level. All the analyses were performed using SPSS 15.0 (IBM, USA) or Prism 5 (GraphPad Software Inc., USA).

Results

Chemerin induces the apoptosis of murine cardiomyocytes

Treatment with 0.1-100 nM chemerin for 48 hours increased apoptosis among HL-1 cells in a dose-dependent manner, as assessed by Hoechst 33258 dye vital staining (Fig. 1A, Panels A.1 and A.2). The increase with respect to controls was statistically significant at both 10 nM (22.24 ± 3.90%; P < 0.05, n=6) and 100 nM (30.29 ± 6.69%; P < 0.05, n=6). In another experiment, using 100 nM chemerin, the difference in apoptosis with respect to controls was shown to increase progressively with time (Fig. 1B, Panels B.1 and B.2) (n=6). A similar dose-dependent effect was obtained with 48 hours’ treatment of neonatal rat cardiomyocytes; once more the apoptosis rates observed with 10 nM (8.15 ± 0.96%; P < 0.05, n=6) and 100 nM (9.70 ± 0.47%; P < 0.05, n=6) differed significantly from those of controls (Fig. 1C, Panels C.1 and C.2).

The induction of apoptosis by chemerin was confirmed by cell-cycle analysis. The chemerin treatment resulted in a (1.22 ± 0.10)-fold increase in apoptosis (P < 0.05, n=6) (Fig. 2A, Panel A.1). In Fig. 2A the Panel A.2 shows, for one of six independent experiments, the percentages of apoptotic, G0/G1-phase and S/G2/M-phase HL-1 cells measured by flow cytometry in controls and after 24 hours’ treatment with 100 nM chemerin; the other five experiments afforded similar results. Cell-cycle analysis likewise confirmed the rise in apoptosis induced by 24 hours’ 100 nM chemerin treatment in neonatal rat cardiomyocytes (Fig. 2B, Panel B.1), showing a (1.72 ± 0.36)-fold increase (P < 0.05, n=6). In Fig. 2B the Panel B.2 shows the raw data from one of six independent experiments.

Chemerin regulates AKT phosphorylation at Thr308 but not at Ser473 in cardiomyocytes

Previous studies have shown that AKT activation reduced apoptotic cardiomyocyte death whereas a decrease in its activity has been linked to apoptosis [35–37]; therefore AKT activation can be considered as a cardioprotective mechanism in cardiomyocytes. According to this, our aim was to study if the apoptosis induced by chemerin could be related to a decrease in AKT phosphorylation in cardiomyocytes.

In neonatal rat cardiomyocytes, 10-100 nM chemerin treatment for 5 minutes or 24 hours induced a dose-dependent decrease in the phosphorylation of Thr308 of AKT, a kinase involved in metabolism, apoptosis and proliferation [38]. At maximum effect (at 100 nM), phosphorylation fell to 0.63 ± 0.05 times control levels for 5 minutes (P < 0.05, n=6) (Fig. 3A, Panel A.1) and to 0.80 ± 0.02 times control levels for 24 hours (P < 0.05, n=3) (Fig. 3B, Panel B.1). Phosphorylation of AKT at Ser473 was not significantly affected (Fig. 3, Panel A.2 and Panel B.2). Similar results were obtained in HL-1 cells, in which 100 nM chemerin treatment showed a significant decrease in Thr308 phosphorylation at 5 minutes (fold decrease 0.79 ± 0.05 vs. control; P < 0.05, n=6) and at 24 hours (fold decrease 0.57 ± 0.12 vs. control; P < 0.05, n=3) (Fig. 4, Panel A.1 and B.1, respectively).

Chemerin induces caspase-9 activation in cardiomyocytes

AKT activation in cardiomyocytes has been shown to inhibit the pro-apoptotic caspase-9, a downstream target of the AKT pathway [39]. In order to evaluate whether the decrease in AKT phosphorylation elicited by chemerin treatment in cardiomyocytes could affect caspase-9 activity and could mediate the induction of apoptosis, we assessed by western blot the cleavage of caspase-9 in cardiac cells treated with chemerin. In HL-1 cardiomyocytes, 10-100 nM chemerin treatments for 24 hours induced a dose-dependent increase in the
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Fig. 1. Results of Hoechst dye vital staining assays of apoptosis in adult mouse atrial HL-1 cells and neonatal rat cardiomyocytes treated with chemerin. Apoptosis in HL-1 cardiomyocytes was revealed by brilliant blue nuclear fluorescence and fragmentation after staining with Hoechst 33258 dye following treatment with 0.1-100 nM chemerin for 48 hours (Panel A.1 and A.2). HL-1 cells were also treated with 100 nM chemerin for 12, 24 and 48 hours. Panel B.1 shows quantitative results (n=6) and Panel B.2 fluorescence microphotographs (X400) of one typical experiment (arrows indicate nuclei with higher fluorescence intensity showing nuclear fragmentation). Panel C.1: treatment for 48 hours with 0.1-100 nM chemerin induced the dose-dependent apoptosis of neonatal rat cardiomyocytes, as assessed by staining with Hoechst 33258; quantitative results (n=6) and Panel C2 fluorescence microphotographs (X400) of one typical experiment. Bar graphs show means ± SEM. *, P<0.05; **, P<0.01.

Cleavage of caspase-9 (Fig. 5). Activation of caspase-9 (39 and 37 kDa fragments) increased 1.35±0.10 times control levels after treatment of cardiomyocytes with 100 nM chemerin (P<0.05, n=6).
Insulin decreases chemerin and CMKLR1 mRNA and protein levels, and reduces chemerin secretion by neonatal rat cardiomyocytes

Since we have observed that chemerin acts as a pro-apoptotic molecule by decreasing AKT phosphorylation in cardiomyocytes, our next goal was to investigate if chemerin expression in these cells could be affected by insulin, a molecule that ameliorates cardiomyocyte survival and prevents the apoptosis induced by oxidative stress through AKT activation in cardiac cells [40, 41].

Treatment of neonatal rat cardiomyocytes with 0.1–100 nM insulin for 24 hours led to a dose-dependent decrease in chemerin mRNA levels, which with the largest dose of insulin fell to 0.67 ± 0.10 times control values (P<0.05, n=6) (Fig. 6A, Panel A.1) The time dependence of this effect was shown in an experiment with 100 nM insulin in which, after an initial lag, chemerin mRNA levels fell progressively to 0.63 ± 0.13 times control values (P<0.05, n=5) after 48 hours (Fig. 6A, Panel A.2). These results were echoed at the protein level, treatment with 100 nM insulin for 24 hours reducing chemerin levels (0.65 ± 0.07)-fold with respect to controls (P<0.01, n=6) (Fig. 6A, Panel A.3 and A.4). Additionally, we determined that neonatal rat cardiomyocytes can secrete chemerin to the culture medium and that its secretion is affected by insulin treatment, as assessed by ELISA. Treatment with 100 nM insulin for 48 hours elicited a decrease of chemerin concentration in culture medium of neonatal cardiomyocytes (7.91±1.32 pg/ml) with respect to controls (13.21±0.59 pg/ml)
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TNF-α modifies chemerin and CMKLR1 mRNA and protein levels in neonatal rat cardiomyocytes

TNF-α is a pro-inflammatory molecule tightly related to physiopathological mechanisms in cardiac diseases [42, 43] that can regulate insulin sensitivity in different cell types [44] and often participates in mechanisms of induction of apoptosis [45], therefore our aim was to investigate if TNF-α could regulate chemerin production by cardiomyocytes.

RT-qPCR analysis showed that treatment of neonatal rat cardiomyocytes for 24 hours with 0.1-20 ng/ml TNF-α elicited a dose-dependent increase in chemerin mRNA levels, with a maximum stimulatory effect of (2.53 ± 0.26)-fold (vs. controls) at 20 ng/ml (P < 0.001, n=6) (Fig. 7A, Panel A.1). The effect was time-dependent, increasing progressively to (5.42 ± 0.44)-
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Fig. 4. Chemerin also reduces the phosphorylation of AKT at Thr308 without affecting Ser473 phosphorylation in HL-1 cells. The decrease of AKT phosphorylation at Thr308 (n=6) (Panel A.1), and the absence of changes in AKT phosphorylation at Ser473 (n=7) (Panel A.2), in chemerin-treated HL-1 cells for 5 minutes were confirmed by western blot. The treatment with chemerin for 24 hours also showed decreased AKT phosphorylation at Thr308 (n=4) (Panel B.1), and no change in phosphorylation at Ser473 (n=4) (Panel B.2). Bar graphs show means ± SEM. *, P<0.05.

Fig. 5. Chemerin treatment stimulates the cleavage of caspase-9 in HL-1 cardiomyocytes. Changes in caspase-9 cleavage (37 and 39 kDa fragments) determined by western blot after chemerin treatment of HL-1 cells for 24 hours (n=6). Bar graphs show means ± SEM. *, P<0.05.
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**Discussion**

The adipokine chemerin is an immune system modulator that acts mainly through its receptor CMKLR1, and that has been associated with metabolic and inflammatory processes [13]. Chemerin and CMKLR1 are produced mainly in the liver and in adipose tissue, but also in a wide range of other tissues (including heart), where it probably can also act as a protein levels was (0.75 ± 0.03)-fold after 24 hours (P<0.05, n=6) (Fig.7B, Panel B.3 and B4). Finally, 100 nM chemerin treatment for 48 hours of neonatal rat cardiomyocytes induced a marked decrease in CMKLR1 mRNA levels (fold decrease 0.61 ± 0.07 vs. control; P<0.05, n=4) (Fig.7B, Panel B.5), suggesting that the induction of chemerin synthesis by TNF-α treatment could mediate the downregulation of the receptor observed in those experiments.
paracrine/autocrine modulator [7, 8] in a variety of functions that includes the regulation of inflammation and metabolism [46]. Chemerin is produced as pre-prochemerin and after the N terminal cleavage is secreted as prochemerin [46]. Previous works have found that prochemerin is the dominant isoform in plasma in healthy subjects and constitute over the 80% of the total chemerin [47], whose concentrations vary widely between species [48] in different pathophysiological conditions [46], involving the extracellular processing of prochemerin a high number of proteases that can also be different in each tissue or in different pathological conditions, which results in multiple chemerin products with different ability to bind to CMKLR1 and exert different functions [6, 46].

Therefore, the experimental and clinical investigations support a role for chemerin in normal physiology and pathophysiology of inflammation and metabolic disorders [11].

That chemerin has a role in cardiovascular disease is supported by chemerin mRNA and protein levels being elevated in epicardial adipose tissue from patients with coronary artery
Ser473 residue. The differences found in the Thr 308 phosphorylation of AKT between affects only Thr308 residue phosphorylation, with no effect on the phosphorylation of phosphorylation in cardiac cells in culture. The decrease in AKT activity elicited by chemerin in the activity of the pro-survival kinase AKT. We demonstrate that chemerin reduces AKT activation, AKT needs to be phosphorylated at yet another site (Ser473) by mTORC2, that protein kinase 1 (PDK1) that leads to an 100-fold increase in AKT activity; for maximal activation, AKT needs to be phosphorylated at Thr308 by 3 phosphoinositide-dependent AKT phosphorylation to be related to apoptosis in various types of cells and regulator located at the crossroads of multiple signalling pathways including metabolism, factors uptake, gene expression and cell to cell communication through autocrine and paracrine functions in myocardium that includes growth, survival, proliferation and also glucose by pressure overload in mice

The main result of the present study is that chemerin can induce apoptosis in cultured cardiomyocytes in a dose- and time-dependent manner (Fig. 1 and 2). The loss of cardiomyocytes has dramatic consequences at cardiac level: the dysregulation of death and survival pathways leading to cardiac myocyte apoptosis in failing hearts is a causal component not only in acute ischemic injury but also in more chronic heart failure states [60, 61] and contributes to cardiac remodelling and contractile failure [62–64]. Apoptosis plays in fact a crucial role in the development of heart failure [65], and previous studies have reported an apoptosis rate ranging from 0.12% to 0.70% in human failing hearts [65]. This small level of apoptosis is considered enough to cause heart failure and even very low levels of apoptosis (23 myocytes/10^5 nuclei) have been shown to be sufficient to induce dilated cardiomyopathy and heart failure [65].

It must also be borne in mind that, although chemerin is a pro-inflammatory molecule, only in rat granulosa cells it has been shown to increase apoptotic rates [66]. Previous works had instead determined that chemerin can reduce the proliferation of human synovial fibroblasts without affecting cell viability [67] and, moreover, it can promote the proliferation of C2C12 myocytes [68].

AKT, also called protein kinase B (PKB) because of its similarity with protein kinase A and C, is a serine/threonine kinase involved in the regulation of a variety of cellular functions, including metabolism, glucose uptake, proliferation, and protein synthesis, all assigned toward the single goal of cell survival [65]. AKT is considered to exert a cardioprotective role; and several evidences support that its activation reduces the apoptosis of cardiomyocytes under ischemia-reperfusion conditions [37], oxidative stress [40] and heart failure induced by pressure overload in mice [62]. AKT substrates exert a decisive influence on cellular functions in myocardium that includes growth, survival, proliferation and also glucose uptake, gene expression and cell to cell communication through autocrine and paracrine factors [18]. In summary, in the myocardial context, the kinase AKT is an important mediator and regulator located at the crossroads of multiple signalling pathways including metabolism, proliferation, differentiation, and apoptosis [18]. Previous studies have found a decrease in AKT phosphorylation to be related to apoptosis in various types of cells [69, 70]. For its basal activation, AKT needs to be phosphorylated at Thr308 by 3 phosphoinositide-dependent protein kinase 1 (PDK1) that leads to an 100-fold increase in AKT activity; for maximal activation, AKT needs to be phosphorylated at yet another site (Ser473) by mTORC2, that further increases AKT activity by 10-fold [65]. Our purpose in this study was to investigate if the apoptosis induced by chemerin in cardiomyocytes could be mediated by alterations in the activity of the pro-survival kinase AKT. We demonstrate that chemerin reduces AKT phosphorylation in cardiac cells in culture. The decrease in AKT activity elicited by chemerin affects only Thr308 residue phosphorylation, with no effect on the phosphorylation of Ser473 residue. The differences found in the Thr 308 phosphorylation of AKT between
HL-1 cells and the neonatal rat cardiomyocytes could be explained by the different origin of both cardiomyocyte models: HL-1 cardiomyocytes are a tumoral cardiac cell line derived from mouse. On the other hand, the neonatal rat cardiomyocytes come from a mixture of cardiomyocytes from atria and ventricles, and are a heterogeneous group of individuals from different gender and progenitors. Despite the inherent heterogeneity of the neonatal rat cardiomyocytes, the decrease in the Thr 308 phosphorylation of AKT induced by treatment with 100 nM chemerin remain statistically significant at 24 hours.

Several studies have investigated the effects of chemerin treatment on AKT phosphorylation: chemerin increases phosphorylation of Ser473 in both human umbilical vein endothelial cells, leading to the activation of endothelial nitric oxide synthase and increased nitric oxide [21, 22], and in human articular chondrocytes, promoting inflammatory signalling [23]; on the other hand, it decreases phosphorylation at Thr308 in both human skeletal muscle cells (in which Ser473 phosphorylation is also decreased) [24, 25] and, in vivo, in mouse skeletal muscle [26], leading in both cases to insulin resistance. Zhang et al. (2014) have recently found that chemerin diminished the glucose uptake by rat cardiomyocytes, and suggested that this effect could be mediated by a negative influence of chemerin on AKT phosphorylation [59], other authors have found that in skeletal muscle of mice chemerin treatment decreased the insulin-stimulated AKT phosphorylation, produced alterations in the mitochondrial function and increased oxidative stress, with implications in the regulation of insulin resistance [57]. On the other hand, the knockdown of the chemerin receptor (CMKLR1) expression reversed the induction of mitochondrial dysfunction elicited by chemerin in skeletal muscle, which provide evidence that chemerin could play an important role in cell viability and metabolism of skeletal muscle via AKT regulation [57].

Although it has been suggested that chemerin-induced apoptosis is mediated by up-regulation of intracellular pro-apoptotic factors (PTEN and caspase-3) and down-regulation of anti-apoptotic factors (PARP and phospho-AKT) in rat granulosa cells [66], the hypothesis that reduced phosphorylation of AKT specifically at Thr308 may also play a role is suggested not only by our present results, but also by the finding that in HEK293 cells the apoptosis inducer staurosporine inhibits AKT phosphorylation only at Thr308, not at Ser473 [71]. Our present finding that in murine cardiomyocytes chemerin treatment reduces the phosphorylation of AKT at Thr308 without affecting Ser473 seems to be consistent with these latter studies (Fig. 3 and 4). Unlike staurosporine, however, chemerin appears not to affect phosphorylation of AMPK, ERK1/2 or p38 MAPK, at least not in the present study of its effects on murine cardiomyocytes (data not shown).

In the AKT pathway, caspase-9 is a downstream target that mediates apoptosis in cardiomyocytes [64]. Our results demonstrate that chemerin also increases the activity of caspase-9, which could have a direct implication in cardiomyocyte apoptosis through the activation of apoptotic mediators of the AKT pathway.

Insulin is a widely recognized protective factor in the cardiovascular system and an activator of PI3K/AKT [41]: previous works have shown that insulin can prevent cardiomyocyte apoptosis induced by oxidative stress [40] and by hypoxia/reoxygenation injury, both of them through activation of the pro-survival PI3 kinase/AKT signaling pathway [72]. We investigated if insulin could affect the expression levels of chemerin (that we show here to be pro-apoptotic in cardiomyocytes) and CMKLR1 in cultured cardiac cells. Our finding that insulin treatment reduced chemerin and CMKLR1 mRNA, protein levels and decreased the release of chemerin by neonatal rat cardiomyocytes (Fig. 6) appears to be the first indication that insulin can act as a negative regulator of chemerin and CMKLR1 gene expression. Our data suggest that insulin and chemerin exert antagonistic roles at cardiac level, which could participate in the mechanisms involved in insulin resistance in cardiomyocytes, in view of the negative effects of chemerin on glucose uptake recently reported by Zhang et al. (2014) and confirmed by our observations (data not shown) [59]. Two previous studies found however that insulin upregulated chemerin levels and secretion by human and murine adipocytes [73, 74], highlighting the tissue-dependent nature of chemerin biology.
TNF-α is a pro-inflammatory cytokine that is closely related to the physiopathology of several cardiovascular diseases through the upregulation of genes that control inflammation, proliferation and apoptosis in cardiomyocytes [75]. Previous works have shown that other adipokines can modulate the effect of TNF-α, particularly the adipokine leptin, a crucial regulator of energy homeostasis that confers protection against the apoptosis induced by TNF-α [45]. According to this, our aim was to investigate if TNF-α treatment could modulate chemerin and CMKLR1 synthesis by cardiomyocytes. In previous studies TNF-α treatment has been found to increase chemerin mRNA and protein levels in 3T3-L1 adipocytes [12], increase serum chemerin levels in mice [12], increase the levels of chemerin mRNA but not CMKLR1 mRNA in visceral adipocytes collected from obese patients [76], and increase both chemerin and CMKLR1 gene expression in human endothelial cells [77]. Given the additional present finding that TNF-α treatment up-regulates chemerin but down-regulates CMKLR1 in murine cardiomyocytes, it is clear that the effect of TNF-α on CMKLR1 gene expression depends on cell type (Fig. 7). After the treatment with TNF-α, we did not detect changes in chemerin secretion by cardiomyocytes (data not shown). We hypothesize that the divergence with the changes induced by TNF-α in chemerin at the mRNA and protein levels could be due to: 1) TNF-α might regulate extracellular proteolytic processes that degrade secreted chemerin into smaller bioactive peptides, that retain biological activity on CMKLR1 but could not be detected by ELISA in the supernatants of cultured cardiomyocytes; and 2) the high levels of chemerin induced by TNF-α could act in an intracrine manner in cardiomyocytes. The down-regulation of CMKLR1 in cardiomyocytes may constitute negative feedback compensating the elevated intracellular increase in chemerin that is induced by TNF-α, as we could confirm with the decrease in CMKLR1 mRNA levels after chemerin treatment of neonatal rat cardiomyocytes (Fig. 7). Other authors have found that, in chondrocytes, chemerin expression can be regulated by factors (such as IL-1β and LPS) implicated in inflammatory processes [78]. More studies are needed to elucidate the regulation of chemerin and CMKLR1 in cardiomyocytes, and the possible relation of the influence of TNF-α on chemerin to cardiac inflammation, cardiomyocyte apoptosis, and cardiac disease, serum chemerin being correlated not only with TNF-α but also with other components of metabolic syndrome and markers of inflammation such as C-reactive protein (CRP) and IL-6 [79], while TNF-α, which is implicated in several cardiovascular diseases, has been reported to mediate inflammation and apoptosis in HL-1 cardiomyocytes [80, 81].

Conclusions

We have shown that chemerin seems to have detrimental effects on cultured cardiomyocytes viability through partial suppression of AKT phosphorylation and subsequent activation of caspase-9; and that chemerin and CMKLR1 levels in cardiac cells are negatively regulated by insulin (a well-known cardioprotective molecule) and positively by TNF-alpha (a molecule known to drive inflammatory processes). All those results suggest that the pro-inflammatory adipokine chemerin could have damaging effects at cardiac level, and that the cardiac chemerin autocrine/paracrine system is regulated by key mediators of inflammation and metabolism.

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Disclosure Statement

The authors have no conflict interests.

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