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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Prolonged leptin treatment increases transient outward K^+ current via upregulation of Kv4.2 and Kv4.3 channel subunits in adult rat ventricular myocytes

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Abstract Circulating leptin levels are elevated in obesity and hyperleptinaemia has been postulated to be an independent risk factor for the development of cardiovascular diseases. Although many studies have been published on the mechanisms involved in the effects of leptin on cardiac function and pathological remodeling, scarce information is currently available analyzing the influence of prolonged leptin treatment on ionic cardiac channels remodeling in adult ventricular myocytes. Enzymatically isolated adult rat ventricular myocytes were treated with leptin or vehicle for 48h. Real-Time RT-PCR were used to analyze mRNA expression of Kir2.1, Cav1.2, Cav 3.1, Kv4.2 and Kv4.3 α-subunits and KChIP2 auxiliary subunit. The fast transient outward potassium channels (I_{tof}) α-subunits Kv4.2, Kv4.3 and KChIP2 were analyzed by Western-blot. The fast transient outward potassium current and the action potentials were recorded in isolated myocytes by the whole-cell patch-clamp technique. Leptin treatment induced an up-regulation of Kv4.2, Kv4.3 and KChIP2 subunits mRNA expression. However, transcriptional levels of Kir2.1, Cav1.2, or Cav3.1 α-subunit channels were unmodified by leptin. Protein expression levels of Kv4.2, Kv4.3 and KChIP2 subunits were also increased by leptin. The electrophysiological study showed that leptin increases the fast transient outward potassium current amplitudes and densities shortening action potential duration. In addition, leptin activated Akt signaling in cardiomyocytes and this mechanism was involved in the effect of leptin on I_{tof} channels. In conclusión, leptin increases both the expression and function of $_{Itof}$ channels in adult ventricular myocytes and this mechanism involves Akt signaling. Altogether these data suggest that leptin could exert beneficial or detrimental effects depending on the initial ventricular myocyte repolarizing reserve.

Keywords leptin · Kv4.2 · Kv4.3 · Akt signaling · cardiomyocytes · transient outward potassium current · ionic channel remodeling

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Introduction

Leptin is an adipocyte-derived protein hormone encoded by the OB-gene that is involved in the central regulation of food intake and energy expenditure [17, 63]. Sources of leptin are

not restricted to adipose tissue, and it is well accepted that myocardial tissue can also produce leptin, exerting autocrine and paracrine effects [2, 52]. In addition to this, leptin receptors OB-Ra and OB-Rb are expressed in the heart supporting a direct effect of leptin on cardiac tissue [41]. Furthermore, obesity, even when uncomplicated by hypertension or diabetes, is frequently associated with hyperleptinemia [13], and high levels of leptin have been postulated as a risk factor for heart failure [55].

Ion channel remodeling associated with myocardial damage is a well-known event that can contribute to cardiac rhythm disturbances in the pathological heart [3, 34, 54]. Studies in animal models and in humans have revealed that the ventricular action potential (AP) prolongation observed in failing hearts increases the risk to suffer arrhythmias and was associated with functional downregulation of ventricular repolarizing K^+ currents [3, 23, 24]. Indeed, changes in the expression of L- and T-type calcium channels have also been related to ventricular hypertrophy and heart failure [28, 40].

The stimulation of leptin cardiac receptor is associated with janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling and also with the activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways which have been implicated in myocyte hypertrophy and cardioprotection [49, 62]. In addition, enhanced PI3K signaling has been associated with upregulation of K^+ currents and improvement of electrical remodeling in pathological hypertrophy and heart failure [58].

During the last years, increasing attention has been paid to understand the mechanisms involved in the effects of leptin on cardiac function and remodeling [2, 42, 52, 56]. However, it remains unknown whether prolonged leptin treatment can modulate ionic channel expression in cardiac tissue. Therefore, the aim of the present study was to investigate the effect of prolonged leptin exposure on the functional expression of the main channels that participate in the repolarizing phase of the ventricular AP in adult ventricular myocytes [35]. We have analyzed the effect of leptin on the fast transient outward potassium channel (I_{tof}) encoded by Kv4.2 and Kv4.3 α-subunits, the inwardly rectifying potassium (Kir) channel encoded by Kir2.1 α-subunit, the L-type calcium channel (I_{CaL}) encoded by the α-subunit Cav2.1, and the T-type calcium channel (I_{CaT}) encoded by the α-subunit Cav3.1. We report here that treatment of rat ventricular myocytes with leptin for 48 h upregulates the transcript and the protein level of the molecular components of I_{tof} channels and increases the amplitude and density of I_{tof}. However, transcription levels of Kir2.1, Cav1.2, or Cav3.1 α-subunit channels were unmodified by leptin treatment. Furthermore, our results demonstrate that the effect of leptin on the expression and function of the I_{tof} channel was mediated by AKT signaling.

Methods

Isolation of adult ventricular cardiomyocytes and experimental design

All experimental procedures were performed in accordance with the animal care guidelines of the European Union (2010/63/EU), and this study conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication No. 85–23, revised 1996). Experiments were approved by the Bioethical Committee of the Complutense University of Madrid.

Rats were heparinized (4 IU/g, i.p.) and properly anesthetized (sodium pentobarbital, 50 mg/kg, i.p.) with analgesia (buprenorfin, 0.05 mg/kg, i.p.). Adequacy of anesthesia was determined by assessing loss of the pedal reflex. Ventricular myocytes were isolated as previously described [46]. Briefly, the ascending aorta was cannulated and the heart was perfused in a Langendorff system with collagenase (251 IU/mL, Worthington type II). Hearts were first perfused for 2–3 min at 36-37°C with a standard Ca^{2+}-free Tyrode solution which contained 0.2 mmol/L EGTA, and then for 3–4 min approximately with the same Tyrode solution containing 251 IU/mL of collagenase type II (Worthington Biochemical) and supplemented with 0.1 mmol/L $CaCl_2$. After perfusion, the hearts were removed from the Langendorff apparatus and the left ventricle, excluding the septum, was cut up, chopped into small pieces, and gently stirred for 2–5 min in a standard Tyrode solution containing 0.1 mmol/L of $CaCl_2$ to disperse the isolated left ventricular myocytes. The resulting cell suspensions were filtered through a 250 μm nylon mesh and centrifuged for 3 min at $20\times g$. The pellet obtained was resuspended in the Tyrode solution containing 0.5 mmol/L $CaCl_2$. Finally, the cells were again centrifuged and resuspended in a Tyrode solution containing 1 mM $CaCl_2$. This procedure yielded quiescent rod-shaped myocytes that were viable for up to 2 days when incubated at 37 °C in Tyrode solution supplemented with 1 mM $CaCl_2$, 10 IU/L insulin, 1 mg/mL BSA, 100 IU/mL penicillin, and 0.1 μg/mL streptomycin [8, 15]. For each experiment, we employed ventricular cardiomyocytes isolated from two rat hearts which were stimulated for 48 h at 37 °C with vehicle (water) or 100 ng/mL of leptin. In some experiments, myocytes were pretreated for 1 h with 1 μM of the AKT inhibitor triciribine. Cells were divided into four groups: one of them was treated with vehicle (DMSO 0.01 %), another one was treated with leptin 100 ng/mL plus vehicle (DMSO 0.01 %), another group was treated with 1 μM triciribine, and finally, another group was pretreated for 1 h with triciribine and then with 100 ng/mL leptin. The leptin concentrations were chosen based on reported values of plasmatic concentration in obese individuals [43, 50] and on previous experimental studies in isolated myocytes [1].

Solutions and drugs

The Ca^{2+}-free Tyrode solution for myocyte isolation contained (in mmol/L): 130 NaCl, 5.4 KCl, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 25 HEPES, 5 $NaHCO_3$, and 22 glucose; pH adjusted to 7.4 with NaOH.

The storage solution for myocytes incubation contained (in mmol/L): 130 NaCl, 5.4 KCl, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 25 HEPES, 5 $NaHCO_3$, 22 glucose, 1 $CaCl_2$, 10 IU/L insulin, 1 mg/mL BSA, 100 IU/mL penicillin, and 0.1 μg/mL streptomycin; pH 7.4.

The solution for I_{tof} current recordings contained (in mmol/L): 135 NaCl, 10 glucose, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$, 5.4 KCl, and 2 $CoCl_2$; pH 7.4. The intracellular recording pipette solution contained (in mmol/L): 125 potassium aspartate, 25 KCl, 10 EGTA, 5 HEPES, 1 $MgCl_2$, 5 ATP, and 0.4 GTP; pH 7.2.

Action potentials were measured in a standard external solution containing (mM): 140 NaCl, 4 KCl, 1.1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH). The internal solution contained (mM): 125 KCl, 4 $MgCl_2$, 5 EGTA, 10 HEPES, 10 glucose, 5 Na_2ATP, and 5 disodium creatine phosphate; pH adjusted to 7.2 with KOH.

Electrophysiological recordings

Whole-cell recordings were obtained at room temperature (22–24 °C) using an Axopatch-200B amplifier (Axon Instruments) interfaced to a Digidata 1322A (Axon Instruments). The recording pipettes were made from 1.5-mm-OD soft-glass capillary tubing by using a P97/PC, Sutter Instruments puller. Pipettes tip resistances ranging from 0.9 to 2 MΩ. Series resistances (R_s) averaged 4.4±0.1 MΩ (n=65) and was compensated by 80 %. Voltage-clamp paradigms were controlled by pClamp 8 software (Axon Instruments).

In the rat, the cardiac transient outward potassium current (I_{to}) is composed of fast (I_{tof}) and slow (I_{tos}) components. I_{to} was evoked by depolarizing pulses from a holding potential of −80 mV to voltage steps from −70 to +60 mV in 10-mV increments for 500 ms; a prestep to −40 mV for 25 ms was used to inactivate sodium current. The pulse interval was 0.1 Hz. I_{tof} was obtained by the subtraction of the currents obtained before and after 3 mM 4-AP application [8, 15].

Membrane capacitance (C_m) was elicited by applying ±10 mV voltage steps from a holding potential of −60 mV, and C_m was calculated according to the equation:

$$C_m = \tau_c I_0 / \Delta Em[1 - I_\infty/(I_0)]$$

where τ_c is the time constant of the membrane capacitance, I_0 is the maximum capacitance current value, ΔE_m is the amplitude of the voltage step, and I_∞ the amplitude of the steady-state current. C_m was significantly higher in leptin-treated cells. C_m averaged 101.6±5.2 pF in control cells (n=22) and 122.0±5.0 pF in cells treated with leptin (n=23); P<0.01.

We carried out current-clamp experiments to analyze APs in eight control and eight ventricular myocytes treated with leptin 100 ng/mL for 48 h. APs were elicited at 10-s intervals by 1.5-fold excitation threshold current pulses of 2.5 ms in duration. After stabilization of the records, ten successive APs were recorded. The parameters of the APs for each cell corresponded to the mean of these ten APs. The AP duration (APD) was measured at 20 %, 50 %, and 90 % repolarization.

The voltage dependence of activation was estimated from the chord conductance according to:

$$G = I/(V_m - V_{rev})$$
$$d\infty(V_m) = G/G_{max}$$

where G is the chord conductance calculated at membrane potential V_m, I is the current magnitude, and V_{rev} is the apparent reversal potential of the current. $d\infty(V_m)$ is the steady-state activation parameter and G_{max} is the maximal value of G.

Assuming a Bolzmann distribution, normalized data points were fitted by a nonlinear curve-fitting program to the following equation:

$$d\infty(V_m) = 1/\{1 + \exp[(V_{50} - V_m)/k]\}$$

where V_{50} is the potential at which the conductance is half-maximally activated, and k is the slope factor describing the steepness of the activating curve.

The voltage dependence of steady-state inactivation for I_{tof} was determined by applying a 2 s conditioning pulse from a holding potential of −80 mV to a potential between −60 and 0 mV) followed by a 500 ms test pulse to +40 mV.

Test current (I) obtained from the different prepulses potentials were normalized to the maximal elicited current (I/I_{max}). The voltage dependence of I_{tof} inactivation could be approximated by a Bolzmann distribution function:

$$I/I_{max} = \{1 + \exp[(V_{50} - V_c)/k]\}^{-1}$$

where V_c is the voltage of the conditioning pulse, V_{50} is the potential at which the current is half-maximally inactivated, and k is the slope factor.

RNA extraction and real-time RT-PCR

The messenger ribonucleic acid (mRNA) level of several K^+ and Ca^{2+} channel subunits was measured by reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted from the cardiomyocytes using TRIzol reagent (Invitrogen), followed by chloroform extraction and isopropanol precipitation. The quantity of RNA was determined spectrophotometrically (Nanodrop, Bio-Rad).

cDNA synthesis was carried out using 1 μg of RNA and QuantiTect reverse transcription kit (Qiagen), in accordance with the manufacturer's instructions.

Quantitative PCR was performed in 96-well plates in a 25 μl final volume that contained 2 μl of cDNA, 12.5 μl of Premix Ex Taq (TAKARA), and 0.2 μl of each forward and reverse primer and 0.35 μl of Probe (TIB Molbiol), or 1 μl of each forward primer, reverse primer and, Probe premix (Applied Biosystems) The amplification program was 95 °C for 30 s followed by 45 cycles of 95 °C for 5 s, 56 °C for 20 s, and 72 °C for 15 s for TIB Molbiol primers, or 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min for Applied Biosystems primers. Each cDNA was amplified in triplicate and a corresponding sample without reverse transcriptase (no-RT sample) was included as negative control.

Primers and Taqman probes for calcium channels were (TIB Molbiol):

Cav1.2 sense 5′-AGCAACTTCCCTCAGACGTTTG-3′, Cav1.2 antisense 5′-GCTTCTCATGGGACGGTGAT-3′, and Cav1.2 probe 5′- 6FAM-CAACAAGACAGGGAACA ACCAAGCGG-BBQ-3′; Cav3.2 sense 5′-CTACGTCGCAT TGTAGACAGCAAG-3′, Cav3.2 antisense 5′-TGGTGA ACACGATGTTGCTTATCTC-3′, and Cav3.2 probe 5′-6FAM-CATGGCAGCCATCCTCGTCAATACTC-BBQ-3′.

Simultaneously, 18 s ribosomal RNA was amplified in a single PCR reaction to normalize the mRNA data. For 18 s rRNA the following primers were used: 18 s rRNA sense 5′-CGCAAATTACCCACTCCCGACCC-3′, 18 s rRNA antisense 5′- GGCTACCACATCCAAGGAAG-3′, and 18 s rRNA probe 5′-Tex-CAATTACAGGGCCTCGAAAGA-BBQ-3′.

Primers and Taqman probes for potassium channels were provided by Applied Biosystems as preoptimized kits: Kv4.2 # Rn00581941, Kv4.3 # Rn00709608, KChIP2 # Rn04224181, Kir2.1 #Rn 00568808, and 18 s rRNA (as internal control) #4310893E.

The expression of the chosen genes was normalized to that of 18 s rRNA using the comparative CT method (ΔΔCT) and expressed relative to control.

Western blot

Cardiomyocytes were homogenized in a buffer containing 320 mM sucrose and 50 mM Tris and IGEPAL 1 % as previously described [15, 45]. The buffer was supplemented with phosphatases and proteases inhibition cocktails (Sigma Aldrich). Then they were centrifuged at 13,000×*g* for 15 min at 4 °C. The supernatants were used for immunoblotting.

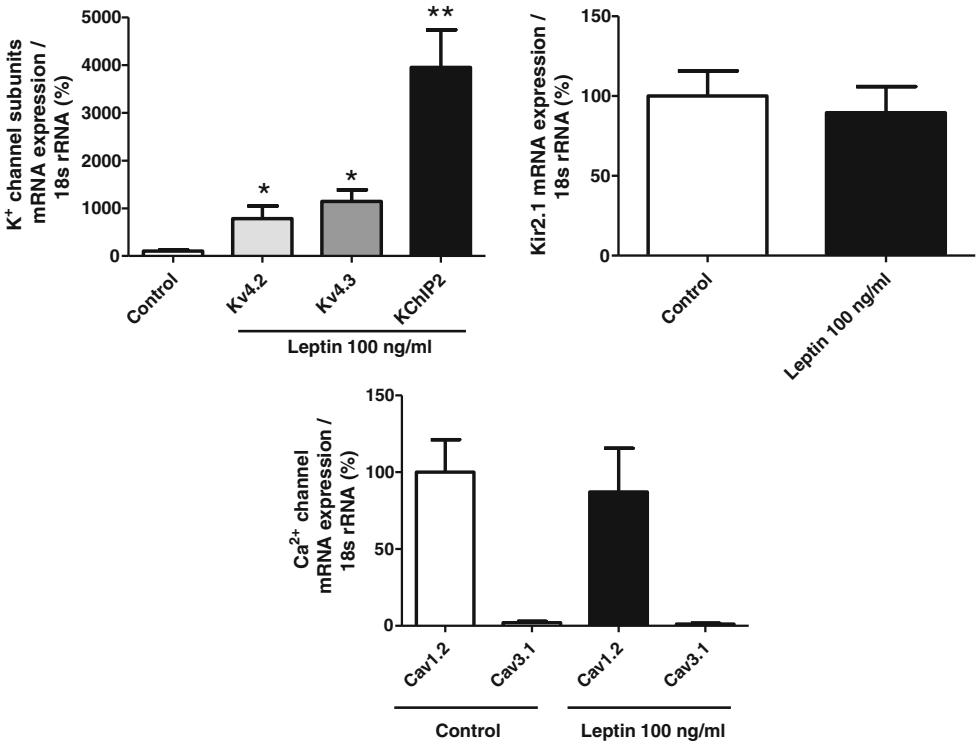

Fig. 1 Expression levels of the transcripts encoding the K^+ and the Ca^{2+} α-channels subunits that underlie rat ventricular action potential repolarization. Channel subunits were measured in individual LV cardiomyocytes samples from control ($n=6$) and leptin-treated myocytes for 48 h ($n=6$). The mean±SEM relative expression levels of Kv4.2, Kv4.3, and KChIP2 channel subunit transcripts were significantly higher in leptin treated than in untreated ventricular myocytes. * $P<0.05$, ** $P<0.01$ vs. control

The extracted proteins were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5 % milk and incubated overnight with primary antibodies: Phospho-AKT (Thr308) and AKT (Cell Signaling), Kv4.2 (Millipore), Kv4.3 (Alomone Labs), the voltage-gated K^+ channel-interacting proteins 2 (KChIP2; Affinity Bioreagents), and GAPDH (Ambion). After incubation with the primary antibodies, blots were incubated with peroxidase-linked secondary antibodies (1:15,000) for 60 min at room temperature. Immunoreactive bands were detected using the Amersham™ ECL™ protein detection system. The intensity of bands was measured with a CCD high-resolution detection system (Chemi Doc; Bio-Rad) coupled with a Bio-Rad personal computer analysis software (Quantity One) or with an Oddysey Fc. detection system (Li-cor).

Statistical analysis

Data are presented as mean±SEM. Statistical comparisons were made using unpaired Student's *t*-test or one-way ANOVA followed by Bonferroni post-test for multiple comparison. $P<0.05$ was considered statistically significant.

Results

Leptin induces transcriptional upregulation of Kv4.2, Kv4.3, and KChIP2 subunits but does not affect Kir2.1, Cav1.2, and Cav1.3 level expression

In the first group of experiments, we have investigated the effects of 48 h exposure of adult myocytes to 100 ng/mL leptin on the expression levels of transcripts encoding ion channel subunits involved in rat ventricular repolarization.

We find higher expression of the subunits encoding I_{tof} channels, Kv4.2, Kv4.3, and Kv channel-interacting protein2 (KChIP2), an auxiliary protein that regulates the expression and/or the properties of ventricular I_{tof} channels (Fig. 1A). In addition, we observed no significant changes in the mRNA levels of Kir2.1, inwardly rectifier potassium channels (Fig. 1B), Cav1.2,

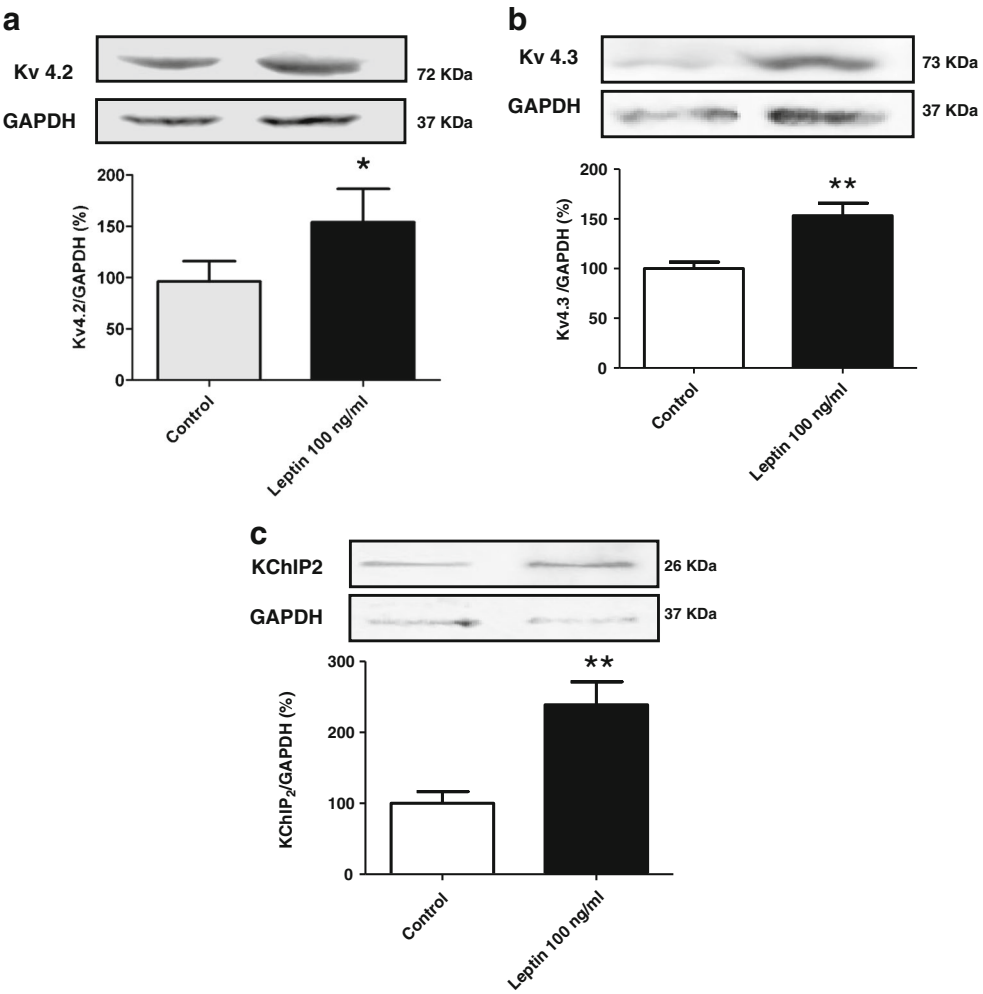

Fig. 2 Leptin induces upregulation of Kv4.2, Kv4.3, and KChIP2 channels subunits. Leptin 100 ng/mL upregulated Kv4.2 (**A**), Kv4.3 (**B**), and KChIP2 (**C**) protein expression. Results are mean±SEM from six to eight independent experiments. * $P<0.05$, ** $P<0.01$ vs. control myocytes treated for 48 h with vehicle

L-type Ca^{2+} channels or Cav1.3, T-type Ca^{2+} channels (Fig. 1C).

Leptin induces upregulation of Kv4.2, Kv4.3, and KChIP2 protein expression

Since leptin upregulates mRNA levels of Kv4.2, Kv4.3 and KChIP2 channel subunits in ventricular myocytes, the next step was to examine the effect of leptin on the protein expression of these subunits. Figure 2A and B shows that 100 ng/mL leptin was also able to increase Kv4.2 and Kv4.3 protein expression in ventricular myocytes. In addition, Fig. 2C shows that KChIP2 was also increased by the long-term leptin cardiomyocyte treatment.

Leptin increases I_{tof} amplitudes and densities and reduces APD without modifying their activation or inactivation properties

The higher expression of Kv4.2/Kv4.3 and KChIP2 channel subunits obtained in cardiomyocytes treated with leptin suggests that the amplitude and densities of the fast transient outward current (I_{tof}) should be increased. Figure 3A shows representative recordings of I_{tof} from two isolated voltage-clamped cardiomyocytes. Depolarizing steps from −10 to + 60 mV from a holding potential of −80 mV, evoked outward currents which were higher in the leptin-treated cardiomyocyte compared with the untreated cardiomyocyte. Figure 3B illustrates the current–voltage relations from control myocytes and

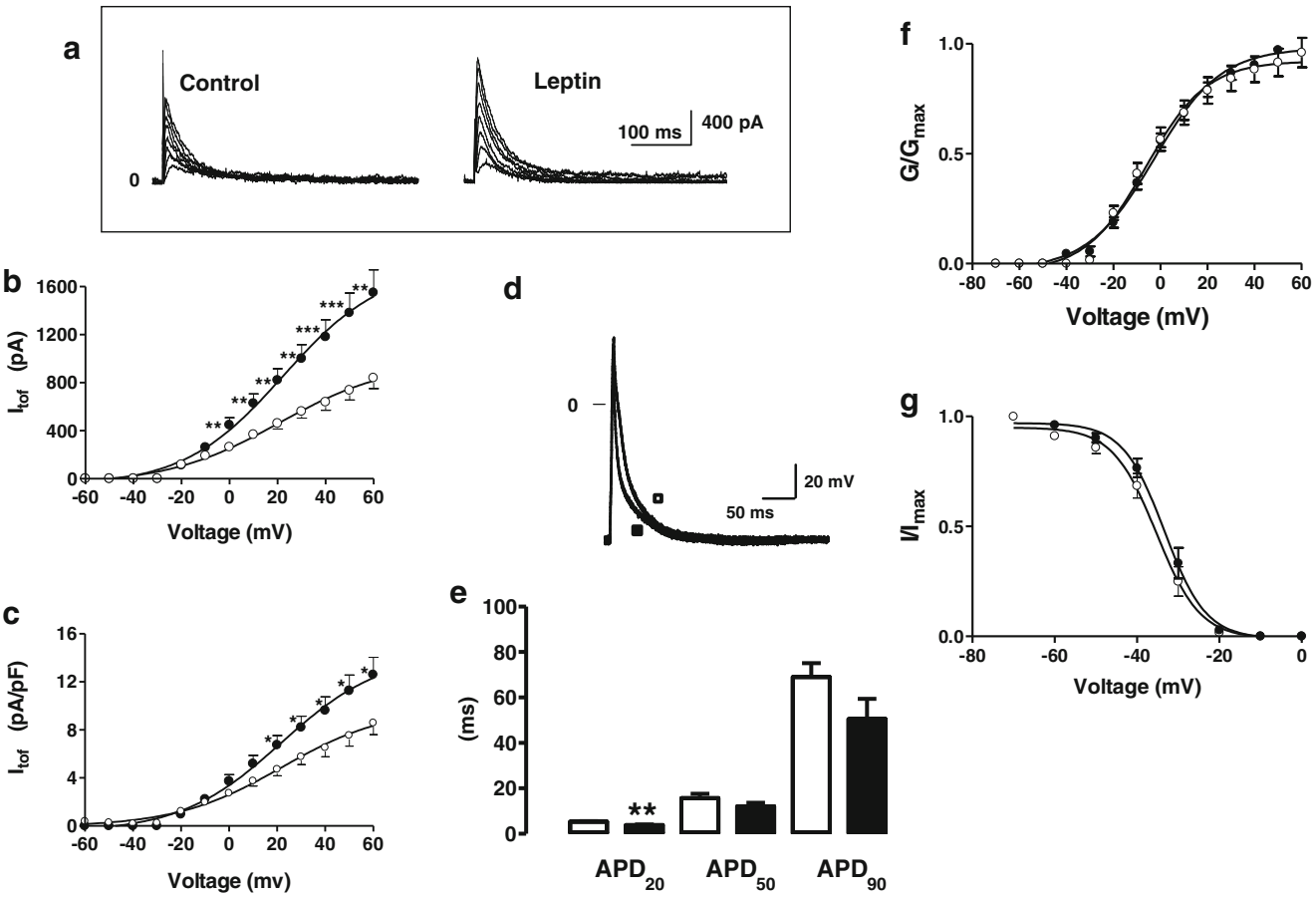

Fig. 3 Leptin increases fast transient outward potassium current amplitude and density in ventricular myocytes and reduces action potential (AP) duration (*APD*) without modifying the voltage dependence of I_{tof} activation and inactivation. **A** I_{tof} traces obtained from −10 mV to + 60 mV in one control cardiomyocyte (*left panel*) and in one myocyte treated 48 h with leptin 100 ng/mL (*right panel*). **B–C** *I–V* relationships for I_{tof} amplitudes (**B**) or I_{tof} densities (**C**) measured in control myocytes treated for 48 h with vehicle (*open circles*; $n=22$) and in myocytes treated with leptin 100 ng/mL for 48 h (*solid circles*; $n=23$). Leptin induced an increase in I_{tof} amplitude and density. **D** Representative superimposed traces of APs recorded in one control myocyte (*open square*) and in one myocyte treated 48 h with leptin (*close square*). **E** Mean APD values measured at 20 %, 50 %, and 90 % of repolarization (APD_{20}, APD_{50}, and APD_{90}, respectively) in eight control ventricular myocytes (*open bars*) and eight ventricular myocytes treated with leptin (*filled bar*). **F** Activation curves obtained in seven control ventricular myocytes (*open circles*) and in eight ventricular myocytes treated with leptin 100 ng/mL for 48 h (*closed circles*). **G** Inactivation curves obtained in six control ventricular myocytes (*open circles*) and in seven ventricular myocytes treated with leptin 100 ng/mL for 48 h (*closed circles*). Results are mean±SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control myocytes

myocytes treated with leptin. The voltage dependence was similar in both groups, but I_{tof} amplitude was significantly higher in cells treated with leptin from 0 to +60 mV. Figure 3C shows current density–voltage relations from control myocytes and myocytes treated with leptin. In this case, the amplitude of I_{tof} was normalized by the membrane capacitance to obtain the current density. The voltage dependence was similar in both groups, but I_{tof} density was significantly higher in cells treated with leptin from +20 to +60 mV which is consistent with the higher expression of Kv4.2/Kv4.3 and KChIP2 subunits obtained in cardiomyocytes treated with leptin. Figure 3D illustrates representative superimposed traces of APs recorded in one control (open square) and in one myocyte treated with leptin (closed square). The APD was shorter in the ventricular myocyte treated with leptin. Figure 3E shows mean APD values measured at 20 %, 50 %, and 90 % of repolarization (APD_{20}, APD_{50}, and APD_{90}, respectively) in control and leptin-treated myocytes. The treatment with leptin induced a significant reduction of APD_{20} (5.3±0.3 ms, $n=8$ in control and 3.8±0.3 ms, $n=8$ in leptin-treated myocytes; $>P<0.01$).

Since the leptin-induced increase in I_{tof} could result from modification of channel properties, the voltage dependence of activation, and inactivation of I_{tof} were measured. Figure 3F shows the normalized conductance (G/G_{max}) vs. membrane potential in control myocytes and in myocytes treated with 100 ng/mL leptin. I_{tof} was activated in both groups at voltages positives to −40 mV and fully activated at +60 mV. Both curves (treated and non-treated with leptin) showed similar values of half-maximal activation voltage (V_{50}, -35.3±1.6 vs. -33.4±1.7 mV in the leptin-treated group) and slope (k, -5.7±0.7 vs. -5.0± 0.2 mV in the leptin-treated group, respectively). Figure 3G shows the voltage dependence of I_{tof} inactivation vs. membrane potential in control myocytes and in myocytes treated with 100 ng/mL leptin. The voltage dependence of I_{tof} inactivation in cells treated with leptin for 48 h was similar to that observed in control cells. The half-maximal inactivation voltage (V_{50}, -35.3±1.6 vs. -33.4±1.4 mV in the leptin-treated group) and the slope factor (k, -5.7±0.7 vs. -5.0± 0.2 mV in the leptin-treated group) were also similar in both groups.

Leptin activates AKT pathway in ventricular myocytes

Previous reports have postulated that the activation of PI3K pathway is related to the upregulation of K^+ channels [57, 58]. Moreover, it has been reported that leptin is able to activate PI3K in the heart [49] and in neonatal rat cardiomyocytes [62]. To explore the implications of this mechanism in the leptin-induced upregulation of the molecular components of I_{tof}, we have investigated whether leptin activates AKT, a well-characterized target of PI3K [31, 48] in cardiomyocytes. When AKT phosphorylation was analyzed after leptin administration, a significant activation of AKT was

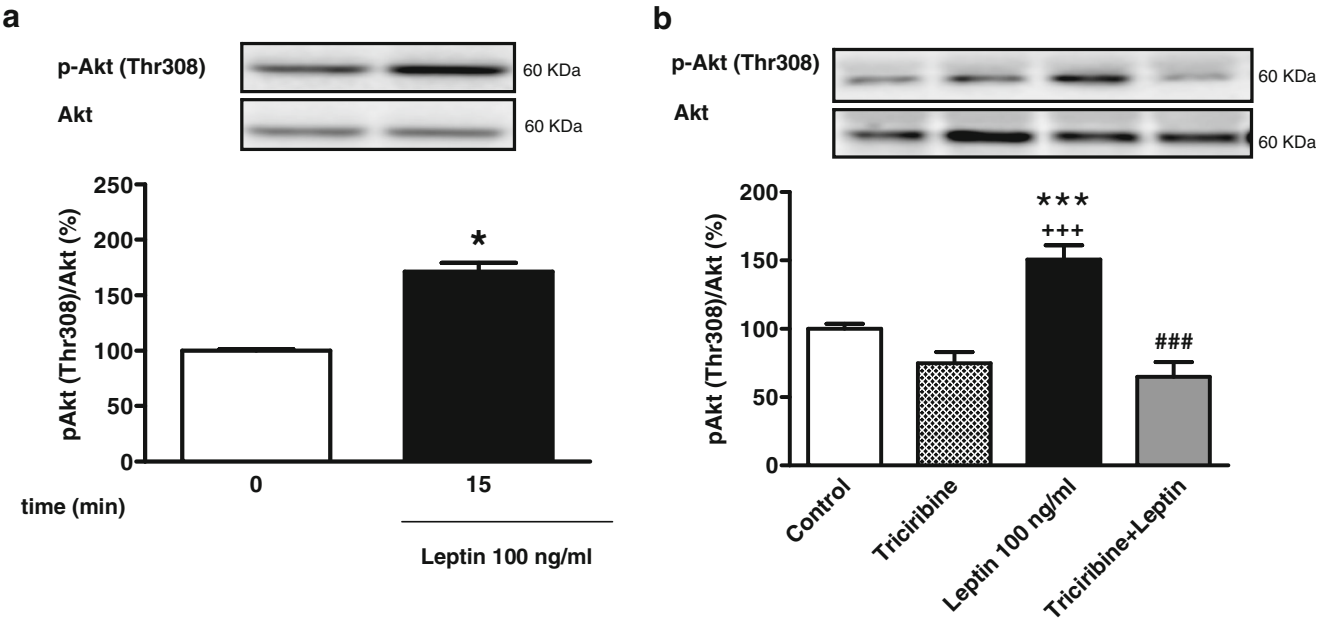

Fig. 4 Leptin activates AKT pathway in adult cardiomyocytes. **A** Ventricular myocytes were exposed to leptin 100 ng/mL for 15 min. A significant activation of AKT was observed after leptin treatment. **B** Pretreatment of myocytes with the AKT inhibitor triciribine (1 μM) completely abolished leptin-induced AKT activation. Results are mean ±SEM from four independent experiments. $*P<0.05$, $***P<0.001$ vs. control, $+++P<0.001$ vs. triciribine, and $###P<0.001$ vs. leptin

observed at 15 min (Fig. 4A). Pretreatment of ventricular myocytes with the AKT inhibitor triciribine [9] completely abolished leptin-induced AKT activation (Fig. 4B).

Leptin induces upregulation of Kv4.2, Kv4.3, and KChIP2 protein expression through the AKT-pathway

After analyzing AKT activation following leptin treatment, we investigated whether the protein expression of the molecular components of I_{tof} was modulated by AKT.

Figure 5A shows that leptin induced a significant increase in Kv4.2 protein expression that was prevented by triciribine. Similar results were obtained for Kv4.3 (Fig. 5B) and KChIP2 (Fig. 5C). Altogether, these results demonstrate that leptin treatment upregulates protein expression of Kv4.2, Kv4.3, and KChIP2 through activation of AKT signaling.

Leptin increases I_{tof} amplitude and density through AKT signaling

To further confirm that AKT activation is involved in the functional modulation of Kv4.2/Kv4.3 by leptin, I_{tof} at +40 mV was recorded in cardiomyocytes pretreated with triciribine and in cardiomyocytes pretreated with triciribine plus leptin. Figure 6A shows similar I_{tof} traces obtained at +40 mV in myocytes treated with triciribine (upper panel) and in myocytes pretreated with triciribine and leptin (bottom panel). The bar graphs in Fig. 6B and C show the mean values of I_{tof} amplitude and I_{tof} density at +40 mV, respectively, obtained in control cells ($n=22$), in cells treated with leptin ($n=23$), in cells only treated with triciribine ($n=7$), and in cells treated with triciribine plus leptin ($n=13$).

The presence of triciribine prevents the effects induced by leptin on I_{tof} in ventricular myocytes.

Discussion

The novel finding of this study is that prolonged leptin treatment upregulates I_{tof} channels through AKT signaling in adult ventricular myocytes. It is well known that the alteration of K^+ channels is a major cause of electrophysiological remodeling in the pathological heart. It

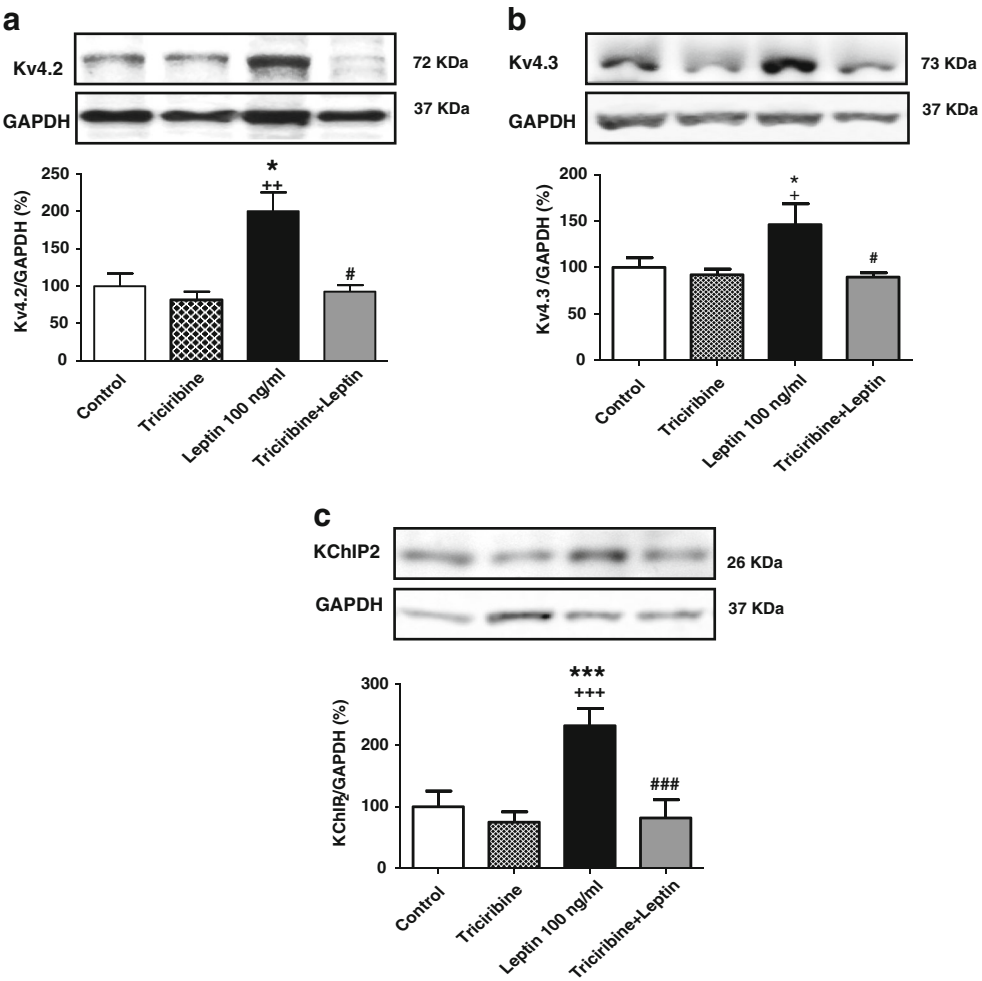

Fig. 5 Leptin induces upregulation of Kv4.2, Kv4.3 and KChIP2 protein expression through AKT-pathway. Treatment with leptin 100 ng/mL for 48 h was able to induce a significant increase of Kv4.2 (**A**), Kv4.3 (**B**) and KChIP2 (**C**) protein expression that was prevented by pretreatment with 1 μM triciribine (**A**–**C**). Results are means±SEM from five independent experiments. $*P<0.05$, $***P<0.001$ vs. control; $+P<0.05$, $++P<0.01$, $+++P<0.001$ vs. triciribine and $\#P<0.05$, $\#\#\#P<0.001$ vs. leptin

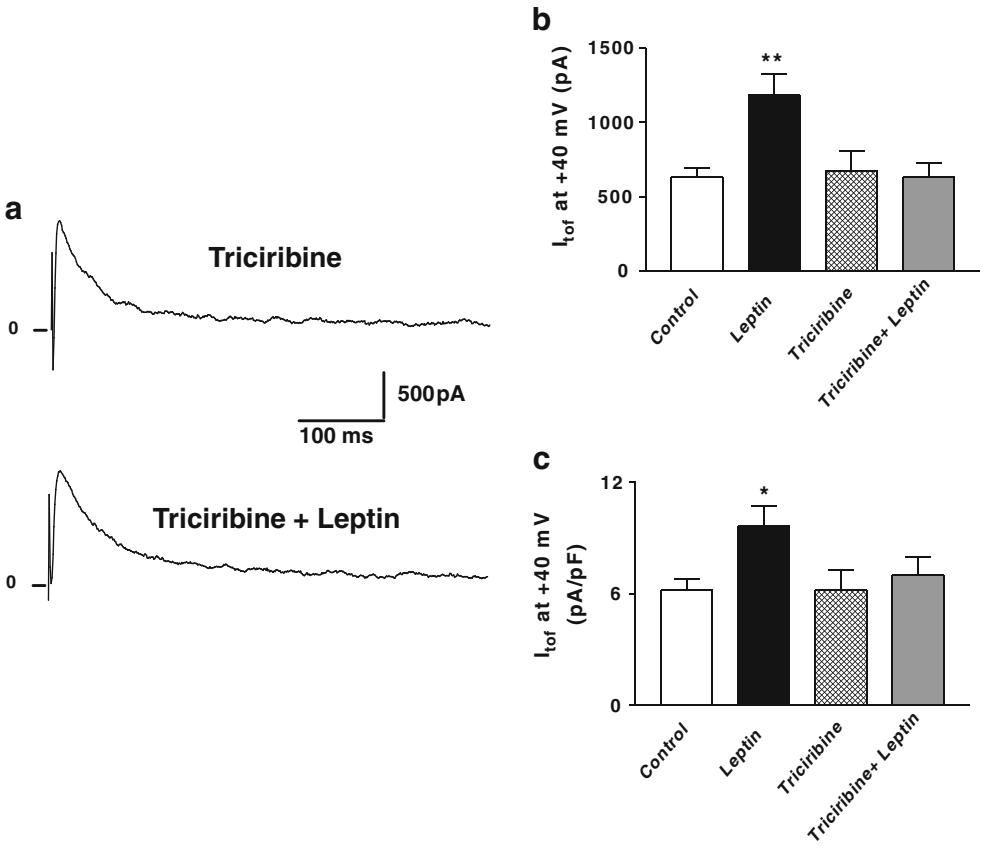

Fig. 6 AKT signaling inhibition prevented the increase in I_{tof} amplitude and density induced by leptin. **A** I_{tof} traces obtained at + 40 mV in one cardiomyocyte treated with 1 μM triciribine and in one myocyte treated with triciribine plus leptin. **B** and **C** Bar graphs showing mean values of I_{tof} amplitude (**B**) or densities (**C**) obtained at +40 mV in control cells (n =22), in cells treated with leptin (n =23), in cells only treated with triciribine (n =7), and in cells treated with triciribine plus leptin (n =13). In the presence of 1 μM triciribine, leptin was unable to modify I_{tof} density and amplitude. Results are means±SEM

has been widely demonstrated that I_{tof}, which plays an important role in determining regional heterogeneity of cardiomyocyte repolarization as well as cardiomyocyte contractility [47], is downregulated in many models of left ventricular hypertrophy (LVH) and heart failure (HF) [7, 10, 18]. In addition to I_{tof} channel remodeling, downregulation of the inwardly rectifying K^+ current (I_{K1}), which plays an important role in the final repolarization phase and in maintaining the stability of the ventricular resting potential, has also been described in LVH and HF [10, 24]. Studies on L-type Ca^{2+} (I_{CaL}) current, the main source of Ca^{2+} entry in cardiac myocytes that contributes to the plateau phase of the AP and is critical for the excitation–contraction process in cardiac myocytes have produced varying results. Some studies in ventricular myocytes of failing hearts have shown a decrease [33, 37], and others no change [26, 32] in I_{CaL} currents densities. In addition, T-type Ca^{2+} channels that are present in neonatal ventricular cardiomyocytes and in pacemaker cells are not detected in adult ventricular myocytes. Nevertheless, T-type Ca^{2+} channels can be re-expressed under pathological conditions [21, 28].

The results of the present study show that mRNA levels and protein expression of Kv4.2/Kv4.3, the α-subunits largely responsible for the I_{tof} in the rat and the auxiliary subunit KChIP2 which is determinant in controlling the cell surface expression of I_{tof} channel [43, 50] are upregulated in ventricular myocytes treated with leptin. However, leptin treatment was ineffective in modulating mRNA expression of other cardiac channel subunits involved in the repolarization phase of the ventricular AP including Kir2.1, Cav1.2, or Cav3.1. Our results show that leptin increases I_{tof} amplitudes and densities shortening APD, without modifying the activation or inactivation properties of I_{tof} channels, reflecting a good correlation between functional and molecular parameters. Several studies [57, 58], but not all [59], have postulated that AKT activation induced by exercise training can upregulate K^+ channels and improve the pathological electrical remodeling associated with LVH and dilated cardiomyopathy. In neurons, upregulation of Kv4.2 subunit by neurotrophic factors, such as neuregulin-1 or neuritin, were reported to be mediated by AKT signaling [60, 61]. In addition, it has been published that leptin is able to activate AKT signaling in cultured neonatal ventricular myocytes [49, 62]. In the present study, we have shown that leptin activates AKT pathway in adult ventricular myocytes, and pharmacological inhibition of this pathway prevented the leptin-induced upregulation of I_{tof} channels. It is well

known that when Akt is activated, it dissociates from the membrane and translocate to the cytosol and nucleus, where it activates downstream signaling pathways. These pathways can mediate Akt long-term effects in protein expression. Then, Akt signaling is terminated by dephosphorylation of Thr308 and Ser473 through the action of protein phosphatase 2 (PP2) and PH domain leucinerich repeat phosphatase (PHLPP) [4, 11]. This regulatory mechanism may explain why the activation of Akt after 15 min of leptin treatment can be enough to mediate long-term effects in our study. Moreover, AKT signaling is involved in the regulation of protein synthesis and cytoprotection of cardiac myocytes [30]. Studies in transgenic mice have confirmed that AKT activation is essential for both cell growth and physiological hypertrophy [29, 48]. Our study demonstrates that the upregulation of Kv4.2 and Kv4.3 α-subunits protein expression induced by leptin is paralleled by upregulation in mRNA expression. Substrates of AKT include glycogen synthase kinase (GSK3β) and forkhead transcription factors (Foxo1 and Foxo3a) [53]. It has been reported that Foxo family regulates the promoter activity of several K^+ channels [39]. GSK3β also modulates the activity of several transcription factors including nuclear factors of activated T-cells (NFAT) promoting their nuclear export [16] which could contribute to the transcriptional regulation of K^+ channels expression [19]. Further studies will be necessary to explore these hypotheses.

In rodents, I_{tof} has a major contribution to phase 1 of ventricular repolarization and APD. In humans, direct contribution of I_{tof} to ventricular APD is limited, although changes in I_{tof} amplitude can alter the amplitude of the plateau phase and thereby indirectly change the kinetics of other transmembrane currents, including I_{CaL} [6]. Therefore, changes in I_{tof} channel expression could modulate excitation–contraction coupling in myocardial cells [47]. We propose that the upregulation of I_{tof} channels induced by leptin could reduce APD, decreasing the duration of Ca^{2+} influx and attenuating cardiomyocyte contractility; however, the consequences of the effect of leptin on I_{tof} channels on cardiomyocytes from pathologic heart could be different. Plasma leptin levels (5–15 ng/mL in lean subjects) are significantly elevated in human obesity (15–500 ng/mL) [38, 43]. However, in obese individuals, leptin usually loses the ability to inhibit energy intake and increase energy expenditure, a phenomenon, referred to as leptin resistance [14]. Nevertheless, there is experimental evidence that leptin resistance would be tissue-dependent and cardiac tissue could be able to preserve full responsiveness to leptin [20, 51]. On the other hand, it is well known that obesity is often associated with pathologies such as diabetes and/or hypertension, providing a status of complicated obesity [5]. This status is characterized by adverse effects on the heart that include pathological cardiac hypertrophy and downregulation of K^+ currents [12, 25, 27]. In this scenario, the mechanism of leptin-induced upregulation of I_{tof} channels that we have shown in our experimental study might have a beneficial effect by compensating the reduced K^+ repolarizing reserve that occurs in pathological hypertrophy associated with hypertension and/or diabetes. Therefore, leptin could protect the pathologic heart from an excessive prolongation of APD, reducing the increased risk of arrhythmias and sudden death in patients with complicated obesity. The possibility that leptin, through this mechanism, could participate in the more favorable clinical outcomes (obesity paradox) [22] seen in obese patients with congestive HF [36] or coronary heart disease [44], compared with normal weight patients, remains as an interesting question to be answered.

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Conflict of interest None

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