IL-6 activates PI3K and PKC-ζ signaling and determines cardiac differentiation of rat embryonic H9c2 cells

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ABSTRACT

Introduction: IL-6 influences several biological processes including the cardiac stem cells and cardiomyocytes physiology. Even if JAK-STAT3 activation is the defining feature of IL-6 signaling, other signaling molecules such as PI3K, PKCs and Erk1/2 are activated eliciting different responses. Moreover, most of the studies on the specific role of these signaling molecules focus on the adult heart and only few data are available on the biological effects evoked by IL-6 in the embryonic cardiomyocytes.

Aim: The aim of this study was to clarify the biological response of embryonic heart derived cells to the IL-6 by analyzing the morphological modifications and the signaling cascade evoked by the cytokine in the H9c2 cells.

Results: IL-6 stimulation determined the terminal differentiation H9c2 cells as evidenced by the increased expression of cardiac transcription factors (NKX2.5 and GATA4,) structural proteins (α-myosin heavy chain and cardiac Troponin T) and the gap junction Connexin 43 protein. This process was mediated by a rapid modification of PI3K, Akt, PTEN and PKCζ phosphorylation levels. PI3K recruitment was an upstream event in the signaling cascade since in presence of PI3K inhibitor IL-6 failed to modify PKCζ, PTEN and Akt phosphorylation, whereas blocking PKCζ activity affected only PTEN and Akt modifications. Finally, the overexpression of a constitutively-active form of PKCζ by H9c2 cells largely mimicked the morphological and molecular effects evoked by IL-6.

Conclusion: This study demonstrated that IL-6 induces the cardiac differentiation of H9c2 embryonic cells process thought a signal cascade that involves PI3K, PTEN and PKCζ enzyme activities.

INTRODUCTION

IL-6 belongs to a family of pleiotropic cytokines that influences a wide range of biological processes involving the stem cells physiology, hematopoiesis, neuronal function, bone metabolism and cardiac physiology (Heinrich, 2003; White, 2011). In the heart, IL-6 is expressed by cardiomyocytes, fibroblasts, vascular endothelial and smooth muscle cells as well as interstitial macrophages (Plenz, 2002; Briest, 2003). IL-6 exerts its function thought the interaction with a signal transduction complex, formed by an 80-kDa ligand-binding subunit, the IL-6 receptor (IL-6R), and by a signal-transducing subunit, the glycoprotein gp130 (Kamimura, 2003). The ligand binding induces gp130 dimerization, the Janus family kinase (JAK) 1 activation, and the phosphorylation and nuclear translocation of the Signal Transducer and Activator of Transcription (STAT) 3 that increases the transcription of target genes. Several studies demonstrated that IL-6 can regulate the expression of cardioprotective factors such as inducible nitric oxide synthase and cyclooxygenase-2 (Tan, 2004; Smart, 2006) and play an important growth-promoting and antiapoptotic role in cardiac myocytes (Kurdi, 2007; Banerjee, 2009). Moreover, the recruitment of the gp130- STAT3 axis plays a pivotal role in directing the transition between compensatory cardiac hypertrophy and heart failure (Baumgarten, 2002; Niebauer, 2000). This process guarantees cardio-protection against physiological and pathophysiological stress by promoting cardiomyocyte survival, inducing compensatory hypertrophy and preserving cardiac function (Hiraoka, 2003; Kanda, 2004; Iemitzu, 2005). Even if STAT3 activation is the defining feature of signaling by IL-6, other signaling cascades are activated and elicit different cellular responses, depending on the cell type. In particular, in cardiomyocytes the gp130 activation involves three major downstream pathways, PI3K, PKCs and Erk1/2, whose synergic or alternative modulation rules the commitment and differentiation, controls maintenance of the phenotype or confers both hypertrophic and cytoprotective responses (Ancey, 2003; Banerjee, 2006; Fredj, 2005; LaFramboise, 2007; Yamauchi-Takihara, 1995; Yin, 2003; Yin, 2006). Therefore, the simultaneous regulation of Akt, PKCs and Erk1/2 pathways by gp130 receptor stimulation appears essential for a balanced

biological outcome in many physiological and pathophysiological settings, especially as these signaling pathways induce partly contradictory or at least conflicting responses. Evidences suggest that Erk1/2 and PKCs play an important role in regulating hypertrophic gene expression and aspects of myofilament remodeling, whereas JAK/STAT activation may be more important in myofilament organization (Wollert, 1996; Kodama, 2000; Wollert, 1997; Bowling, 1999). PI3K/Akt plays an important role in adaptive hypertrophy (Aoyagi, 2011; Weeks, 2012) and Erk1/2 and PI3K play a critical role in cardiomyocyte survival, protecting cardiomyocytes against apoptosis induced by a variety of stress inducers (Negoro, 2001; Smart, 2006). However, the identification of the relative contributions of Erk1/2, PI3K, PKCs and JAK/STAT signaling to cardiomyocyte physiology is complex. Moreover, most of the studies on the specific role of these signaling pathways focus on the adult heart and only few data are available on the biological effects evoked by IL-6 in the embryonic cardiomyocytes.

The H9c2 cell line was originally derived from embryonic rat ventricular tissue (Kimes and Brandt, 1976). Although H9c2 cells are no longer able to beat for their inability to elicit well defined sarcomeres and all the elaborate contractile apparatus, they still show many similarities to primary cardiomyocytes, including membrane morphology, G-signaling protein expression and electrophysiological properties (Sipido and Marban, 1991). For these reasons, they have emerged as an excellent in vitro alternative model to study the primary cardiac differentiation. When exposed to a low concentration of serum H9c2 cells have been reported to differentiate (Di Giacomo, 2010). Although the factors involved in the differentiation of H9c2 cells are still unknown, evidence suggests an involvement of PKC enzymes (Di Giacomo, 2010).

The aim of this study was to evaluate the biological response to IL-6 exposure of embryonic cardiomyocytes. For this reason we analyzed the morphological effects, the molecular responses and the signaling pathway evoked by IL-6 in H9c2 cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) and cell culture media were purchased from EuroClone (Milano, Italy); mouse recombinant Interleukin-6 (IL-6) and PI3K inhibitor LY 294002 were purchased from Sigma Chemical Co. (St. Louis, MO, USA); enhanced chemiluminescence (ECL) kit from Amersham (Little Chalfont, Buckinghamshire, UK); haematoxylin and eosin solutions were from Bioptica (Milano, Italy) and PKCζ pseudosubstrate inhibitor Myristoylated was purchased from Calbiochem (Billerica, MA, USA).

Anti-cardiac myosin heavy chain (αMHC) antibody, anti-cardiac troponin T (cTnT), anti-Nkx2.5, anti-JAK1, anti-pJAK1 (Tyr1022), anti-PKCα, anti-pPKCα (Ser657), anti-PKCδ, anti-pPKCδ (Thr507), anti-PKCε, anti-pPKCε (Ser729), anti-IL-6Rα, anti-gp130 and anti-Connexin 43 (Conn43) antibodies were purchased from Santa Cruz (Heidelberg, Germany); anti-PKCζ, anti-pPKCζ (Thr410), anti-PI3Kp85α, anti-pPI3Kp85α (Tyr607), anti-STAT3, anti-NFkBp65, anti-GATA4, anti α-HA, and HRP conjugated secondary antibodies were from Abcam (Bristol, United Kingdom); anti-pSTAT3 (Ser727), anti-pSTAT3 (Tyr705), anti-Akt and pAkt (Ser473), anti-no pPTEN were from Cell Signaling (Danvers, MA), while anti-PTEN antibody and Alexa Fluor 488 anti-mouse or anti-rabbit were from Invitrogen (Life Technologies, Bethesda, MD, USA). Anti-α-HA tag antibody fluorescein-conjugated was purchased from Roche (Milano, Italy).

Cell culture and manipulation

The H9c2 rat cardiomyoblast cell line was cultured in proliferating conditions (Prol: high glucose DMEM, supplemented with 10% FBS, 2 mM glutamine and 1% penicillin/streptomycin) or in differentiation medium (Diff: high glucose DMEM, 2% FBS, 2 mM glutamine and 1% penicillin/streptomycin) in absence or presence of IL-6 (10 ng/ml) up to 3 days. When required, cells were pre-incubated with 5 μM of the PI3K inhibitor LY294002 or with 10 μM PKCζ

pseudosubstrate inhibitor Myristoylated for 1h and subsequently treated as required.

For transient transfection assays, $4x10^6$ H9c2 cells were electroporated using the Electro Square Porator ECM 830 (BTX, Holliston, MA) with 30 µg of expression vector containing either a catalitically active (caPKCζ) or a inactive (iPKCζ) form of PKCζ inserted in the pHACE backbone with a C terminal Influenza Virus Hemagglutinin (HA) tag of nine amino acids (Soh, 1999 aggiungere lavoro sugli eritroblasti con migliaccio). Cells were analyzed 36 hr after the transfection. Transfection efficiency was calculated on the basis of the number of fluorescent cells obtained in parallel samples transfected with a 30 µg of control plasmid containing pGFP (Clontech Laboratories, Mountain View, CA), as determined with Cytomics FC500 (Beckman Coulter srl). Necrotic cells were excluded from the analysis by 7AAD staining. Thirthy-six hours after transfection, the percent of GFP positive H9c2 cells was 60±8%.

Hematoxylin/ eosin staining and immunofluorescent analysis staining

For the morphological evaluation, 0.5×10^6 cells were plated and cultured on Chamber Slides (LAB TEK, Thermo Fisher Scientific Inc., Waltham, MA, USA) and stained with hematoxylin/ eosin as previously described (Ghinassi, 2010). For the immunofluorescent analysis cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100; after the blocking, cells were incubated with primary antibodies overnight at 4°C and then with the opportune secondary antibody. Nuclei were counterstained with Dapi and slides mounted with Slow Fade Antifade Kit mounting medium. As internal controls, experiments were performed also in absence of the primary antibody. Images were acquired by means of a ZEISS AXIOSKOPE light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Coolsnap Videocamera (Roper Scientific Photometrics, Tucson, AZ, USA) and analyzed with the MetaMorph 6.1 Software (Universal Imaging Corp, Bedford Hills, NY, USA), as described (Verrucci, 2010).

Immunoblot analysis

Cells lysates in RIPA homogenization buffer were electrophoresed in SDS-PAGE and transferred. After incubation overnight at +4°C with the specific primary antibody, membranes were reacted with the adequate HRP- conjugated secondary antibodies. Protein expression was quantified by densitometry with ImageJ Software, as previously described.

Statistical analysis

All quantitative data are presented as the mean ± SD. Statistical comparison was performed using one-way analysis of variance (ANOVA) and Student's *t*-test. The level of significance was set at $p \leq 0.05$.

RESULTS

IL-6 stimulation determines morphological changes and induces phenotypic markers of cardiac differentiation in H9c2 embryonic cells

As evidenced in figure 1A, H9c2 cells growing in proliferation medium (Prol) were characterized by a roundish or stellate shape and expressed low levels of late cardiac differentiation markers such as αMHC, cTnT and Conn43. As expected, H9c2 cultured for three days in low serum condition (Diff) appeared as a heterogeneous population, with some enlarged and more elongated cells, rarely fused to form binucleated cells expressing higher levels of αMHC and Conn43. Interestingly, the addition of IL-6 to the Diff medium determined more important modifications: cells diffusely acquired a more differentiated phenotype, becoming more elongated and several binucleated syncytia were observed; moreover, immunofluorescent analysis evidenced that in presence of IL-6, cells displayed higher levels of αMHC and cTnT that appeared organized in oriented microfilaments. A dramatic induction of Conn43 expression was also detected.

To better characterize the differentiation process induced by the IL-6 treatment, an immmunofluorescent analysis for transcription factors involved in cardiac differentiation was

performed. As shown in figure 1B and in Table I, the reaction for Nkx2.5 and GATA4 evidenced a finely dotted positivity, mainly scattered at cytoplasm levels already in Prol conditions. No modifications were observed when cells were switched in Diff up to 30 min (not shown), whereas the addition of IL-6 determined an increase of the fluorescent intensity that spotty marked both cytoplasm and nuclei. These data suggest that IL-6 treatment early induces the expression of Nkx2.5 and GATA4, together with their translocation into the nuclear compartment.

IL-6 treatment rapidly induces the IL-6R synthesis and the gp130 recruitment to the plasma membrane

IL-6 receptor complex consists of a membrane-tethered gp80, the IL-6R, which binds to the co-receptor gp130, a glycoprotein that bears the signal-transducing domain. Western blot and immunofluofrescent analyses evidenced that H9c2 cells express basal levels of both IL-6R and gp130 (Figure 2) that did not modify when cells were switched up to 30 min in the differentiation medium (Diff) (data not shown). On the other hand, the addition of IL-6 triggered a rapid increase of IL-6R signal evident after only 10 min of treatment both at cytoplasmic and perinuclear levels. After 30 min the IL-6R was still overexpressed, even if at lower extent, whereas after 3 days of IL-6 treatment the differentiated cells displayed expression levels similar to that observed in the proliferating population. On the other hand, the IL-6 exposure did not affect the gp130 protein expression; nevertheless, a reinforced signal at plasma membrane was observed after 10 min, but not after 30 min of IL-6 treatment, suggesting a rapid and transient recruitment of the co-receptor to the cellular membrane (Figure 2).

The PI3K/Akt and PKCζ signaling pathways, but not Erk1/2, are involved in IL-6 induced H9c2 differentiation in cardiomyocytes

It has been reported that binding of IL-6 to its receptor recruits the Janus family kinases that in turn activate three downstream cascades including STAT3, ERK and PI3K/Akt (Ancey, 2003; Banerjee, 2006; Yin, 2006; Fang, 2003). We analyzed the role of these signaling pathways in the IL-6 induced H9c2 myocardial differentiation (Figure 3, panel A). As expected, IL-6 exposure induced a rapid increase of JAK1 and STAT3 phosphorylation levels, sill evident after 30 min. In particular, IL-6 treatment increased the STAT3 phosphorylation levels of the Tyrosine residue at position 705 (Y705), whereas we did not detect any substantial modification at Serine residue at position 727, which represents the substrate for the Erk1/2 and p38 mitogen-activated protein kinase (MAPK) family members (not shown). Accordingly, we failed to find a modification of Erk1/2 expression and phosphorylation levels. IL-6 exposure determined also a modulation of the PI3K, whose phosphorylation levels incremented after 10 and 30 min of treatment. Interestingly, we found a concomitant reduction of Akt phosphorylation level. For this reason, we analyzed the effect of the IL-6 treatment on the Phosphatase and Tensin Homologue (PTEN), the phosphatase that opposes PI3K function leading to the inactivation of Akt. As compared to control cells, H9c2 exposure to IL-6 determined a decrease of Ser380 phosphorylation of PTEN, which unmasks the PDZ enzymatic domains responsible for the Akt de-phosphorylation. No modifications of STAT3, Erk, PI3K/Akt and PTEN were observed when cells were switched in Diff in absence of IL-6 up to 30 min.

It has been already reported that H9c2 cells express PKCα, δ, ε and ζ (Xu, 2009). We thus examined the possible PKC involvement in the signal transduction pathway activated by IL-6 stimulation. Cell lysates were analyzed by Western blot using phospho-specific PKC antibodies (Figure 3, panel B). The expression of PKCα, δ, ε and ζ was detected in unstimulated H9c2 cells with a basal phosphorylation level. Incubation of cells with IL-6 did not affect both the PKCα, δ and ε expression or phosphorylation levels (Ser657, Thr507 and Ser729 for PKCα, δ and ε respectively), whereas determined a rapid increase of PKCζ phosphorylation (Thr410, pPKCζ) still

evident after 30 min of IL-6 exposure. This observation was confirmed by the immunofluorescent analysis that evidenced an increase of the pPCKζ signal both at cytoplasmic and nuclear level after 10 and 30 min of IL-6 treatment (Figure 3, panel C).

PI3K inhibition blocks the IL-6 induced modifications of PKCζ, Akt and PTEN phosphorylation levels

We next investigated the role of PI3K on other components of the IL-6 signaling cascade by pre-treating H9c2 cell with the PI3K inhibitor LY294002 (Figure 4). As expected, PI3K inhibition did not affect STAT3 activation (not shown), but blocked the IL-6 induced PKCζ phosphorylation so that the pPKCζ/PKCζ ratio remained similar to that observed in the control. Moreover, in LY294002 pre-treated cells we failed to detect the IL-6 induced de-phosphorylation of both PTEN and Akt (Figure 4). These results suggested the PI3K requirement for the IL-6 dependent PKCζ and PTEN activation and for the Akt de-phosphorylation.

PKCζ activity is required for the IL-6 induced modifications of PTEN and Akt phosphorylation levels

In order to evaluate the PKCζ role in the differentiation pathway activated by IL-6 in H9c2 cells, we pre-incubated the culture with a cell permeable pseudo-substrate that specifically inhibits PKCζ activity. As reported in Figure 5, IL-6 triggered the PI3K phosphorylation also in presence of the PKCζ inhibitor. Indeed, we observed a similar increment of pPI3K/PI3K ratio after 10 min and 30 min of IL6 treatment in H9c2 cells cultured both in presence and in absence of the PKCζ pseudo-substrate. On the other hand, the PKCζ inhibition affected the PTEN and Akt modification in response to IL-6 treatment. Indeed, in H9c2 pretreated with PKCζ pseudo-substrate the addition to IL-6 did not evoke the reduction of both PTEN and Akt phosphorylation levels that we observed when the isozyme was not inhibited (Figure 5).

These data suggested that in this model IL-6 treatment triggers the PI3K activation upstream the PKCζ recruitment and that PKCζ activity is required for the PTEN activation and Akt de-phosphorylation.

Overexpression of a constitutively-active of PKCζ mimics the effects of IL-6 in H9c2 cells

In order to confirm this hypothesis, expression vectors containing the HA-tagged caPKCζ (costitutively active form), and the iPKCζ (kinase inactive, as internal control) were transiently transfected into H9c2 cells (Figure 6A). The transfection efficiency measured 36hr after the electroporation was of 60±8%. As shown by Western blot analysis performed with the anti-tag, H9c2 cells transfected with caPKCζ and iPKCζ produced comparable levels of the ectopic proteins (Figure 6A). Thirty-six hours after the transfection, H9c2 transfected with caPKCζ developed a more elongated shape and were prone to fuse forming binucleate sincithya; these morphology changes were accompanied by an increase of αMHC and cTnT (Figure 6B), with a fibrillar organization particularly evident in the binucleated cells. Moreover, an induction of GATA4 and Nkx2.5 expression and their nuclear translocation was also observed (Figure 6C). In parallel experiments, when H9c2 cells were transiently transfected with vectors encoding the iPKCζ no modification in cell morphology and in cardiac markers expression and localization were observed. Finally, we investigated the effects of the PKCζ expressing vectors on the other molecules involved in the IL-6 signaling pathway. In caPKCζ transfected cells Western blot analysis evidenced significantly higher no-pPTEN and lower pAkt levels relative to those observed in the control samples. On the other hand, in iPKCζ transfected cells no modification of no-pPTEN was observed, whereas a slight increase of pAkt was detected (Figure 6D).

DISCUSSION

The main findings of this study are that: i. in H9c2 cells IL-6 triggers a cardiac differentiation process by activating specific nuclear factors such as Nkx2.5 and GATA4, and inducing the expression of sarcomeric proteins; ii. these effects are at least partially mediated by a signal cascade that involves PI3K, PTEN and PKCζ enzyme activities; iii. PKCζ mimes the differentiating effect of IL-6. IL-6 influences a wide range of biological processes that also include the cardiac stem cells and cardiomyocytes physiology. IL-6 utilizes a ligand-specific receptor in combination with the co-receptor unit gp130 and mediates its effects by JAK/STAT pathway, whose signaling plays a central role in cardiac physiology and pathophysiology [I.S. Harris, S. Zhang, I. Treskov, A. Kovacs, C. Weinheimer, A.J. Muslin Raf-1 kinase is required for cardiac hypertrophy and cardiomyocyte survival in response to pressure overload Circulation, 110 (2004), pp. 718–723]. Basic research has provided overwhelming support for the conclusion that the IL-6 and STAT3 have beneficial effects on cardiac myocytes. Several reports evidenced, indeed, that the IL-6-gp130-STAT3 axis induces a compensatory hypertrophy and prevents heart failure influencing the remodeling processes (Baumgarten, 2002; Niebauer, 2000). Further evidence suggests an IL-6 growth-promoting and anti-apoptotic role in cardiac myocytes (Banerjee, 2009). Our findings extend the IL-6 role in cardiomyocyte physiology demonstrating that this citokine promotes the cardiac differentiation of embryonic cells, as evidenced by the morphological changes and by the increased expression of Conn43 and of the sarcomeric proteins. These events are preceded by the modification of Nkx2.5 and GATA4 transcription factors, molecules essential for cardiac embryogenesis, since they regulate genes encoding structural and regulatory proteins characteristic of cardiomyocytes. Our findings evidencing that IL-6 induces the expression and the nuclear translocation of these proteins, suggest that the IL-6 differentiating effects on embryonic cells is at least partially mediated by the activity of these cardiac transcription factors. It is worth noting that IL-6 treatment is also accompanied by a rapid up-regulation of IL-6R signal at cytoplasmic and perinuclear levels. This prompt response, evident after only 10 minutes of treatment, is probably

due to the translation of pre-existing mRNA molecules; nonetheless, a synthesis due to the transcriptional activation by specific nuclear factors cannot be ruled out.

Even if JAK-STAT3 activation is a defining feature of the IL-6 signaling, other signaling pathways can be activated including the PI3K/Akt pathway, PKCs and MAP kinases, molecules involved in the regulation of several biological process (…..). However, which pathways are activated and the relative intensity of their activation are very much cell type- dependent. In the adult heart the simultaneous regulation of Akt, PKCs and Erk1/2 pathways by gp130 stimulation appears essential for a balanced biological outcome in many physiological and pathophysiological settings, especially as these signaling pathways induce partly contradictory or at least conflicting responses. Our results evidence that in H9c2 cells IL-6 treatment determines the PI3K and PKCs, but not Erk1/2 kinase activation. This finding is supported also by the observation that STAT3 was phosphorylated at the Tyr705, but not at Serine residue at position 727, which represents the substrate for the Erk1/2 and p38 mitogen-activated protein kinase (MAPK) family members. PI3K-mediated PIP_3 production usually leads to the activation of Akt, the kinase that mediates several of the well-described PI3K responses, mainly growth, metabolism and survival (Manning and Cantley, 2007). Therefore, the PI3K-Akt axis is considered the canonical PI3K signaling. Nonetheless, PI3K leads to the modulation of other important pathways. Interestingly, in our model IL-6 treatment determined an increase of the pPI3K levels together with a reduction of the Akt phosphorylation. This finding suggests that in cardiac embryonic cells Akt signaling might not be regulated solely by PI3K. This is unsurprising since it has been already reported that PI3K regulates skeletal differentiation of H9c2 cells mainly through an Akt-independent pathway (Kim, 1999). Moreover, previous data evidenced that the Akt signaling is strictly regulated during the H9c2 cardiac differentiation by the modulation of specific Ser/Thr phosphatases (Kageyama, 2002). In particular, we evidenced that the IL-6 treatment elicited in H9c2 cells a rapid PTEN de-phosphorylation and activation. This lipid phosphatase blunts the PI3K-Akt signaling axis buffering the AKT activation. Previous findings have already evidenced that, even if in other cell line, IL-6 can cause only a slight increase in Akt

phosphorylation and can induce the PTEN expression (Tassidis, 2010). Moreover, our observations corroborate and extend earlier results evidencing a prominent role for PTEN activity in cardiac differentiation. It has been described, indeed, that the histone deacetylase inhibition that mediates the cardiac differentiation of mouse embryonic stem cells and of H9c2 cells (Chen, 2011; Majumdar, 2012) induces a PTEN specific gene network with the induction of PTEN expression and concomitant reduction in Akt phosphorylation (Majumdar, 2012; Majumdar, 2011). Several reports demonstrated that PTEN activity is tightly controlled by PI3K pathway through a feedback mechanism (Carracedo and Pandolfi, 2008). Accordingly, we observed that in our model the PTEN involvement was a signal event positioned downstream to the PI3K involvement, since the inhibition of PI3K activity with LY294002 abolished the PTEN de-phosphorylation. Moreover our results suggest that in the IL-6 dependent signaling pathway, PTEN activation is positioned downstream also to the PKCζ recruitment: indeed, treatment of H9c2 cells with a specific PKCζ pseudosubstrate prevented the modification of PTEN phosphorylation levels.

Another important finding of this study is that the H9c2 differentiation induced by IL-6 involves PKCζ. We evidenced that H9c2 express PKCα, δ, ε and ζ isoforms, but the latter was the only modulate by the IL-6 treatment. Similarly to what observe in murine cardiomyocytes for PKCβ_2 (Wang, 2006) and in H9c2 for PKCε (Perrelli, 2013), also in our model PI3K appears to be an upstream regulator of PKCζ, since in presence of a PI3K inhibitor IL-6 failed to increase the PKCζ phosphorylation levels. As already reported, indeed, the PI3K pathway is not limited to the regulation of the canonical Akt cascade, but the PIP_3 production can mediate the activation of PKCζ (Randall, 2006). Several reports indicate that PKC enzymes are important regulators in cardiomyocytes and H9c2 cells. There is evidence about the survival role of the cytosolic PKCs pool and of the mitochondrial PKCε (Perrelli, 2013). Moreover, Di Giacomo et al. (2010) evidenced that PKCδ is involved in H9c2 differentiation in skeletal myotubes and that the transfection of PKCδ siRNA to H9c2 cells retards the differentiation process. In this biological model, the

phosphorylated form of PKCδ increased and localized within the nucleus (Zara, 2011) where probably interacts with the transcription machinery in order to determine the morphological modifications related to the differentiated phenotype. Similarly, we observed that upon IL-6 treatment the pPKCζ localized both at cytoplasmic and nuclear levels. It is possible that the involvement of different PKC isoforms (δ or ζ) might represent a key step able to address the differentiation process toward the skeletal or cardiac lineage, respectively.

The PKCζ recruitment seems to be crucial step in the differentiation pathway activated by the IL-6 treatment: inhibition of the PKCζ activity with a specific pseudo-substrate, even if did not affect the PI3K activation, buffered the PTEN and Akt response to IL-6. Moreover, the overexpression of the catalytically active form of PKCζ mimicked the morphological and molecular events elicited by the IL-6 treatment: transfected cells, indeed, displayed significantly higher levels of the active PTEN, that blunted the Akt signal by reducing its phosphorylation; in addition, the gain of function of PKCζ determined changes in the cellular shape, modifications of GATA4 and Nkx2.5 expression and localization and the increase of the cardiac sarcomeric proteins whose fibrillar organization was particularly evident in the binucleated cells.

In conclusion, our study demonstrates that IL-6 treatment determined the terminal differentiation of rat embryonic cardiomyocytes and that this effect is mediated by PI3K, PTEN and PKCζ activities. By inhibiting alternatively PI3K or PKCζ and by overexpressing PKCζ, we have identified a possible signaling pathway that links the activated IL-6 receptor complex and the intracellular signaling mediators (scheme in Figure 7). In our hypothesis IL-6 determines the PI3K activation, that acts as an upstream regulator of PKCζ, which in turn influences PTEN and Akt phosphorylation levels. Many questions remain regarding the IL-6 activated pathway in H9c2: even if the molecular relationships between PI3K and PKCs have been well characterized and already discussed in literature, the signaling events that link the PKCζ and PTEN are less clearly

understood. Nonetheless, our data seem to suggest that PKCζ may activate intermediate molecules responsible for the PTEN de-phosphorylation and activation.

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FIGURES LEGEND

Figure 1: IL-6 determines important morphological modifications of cell shape and induces specific myocardial markers expression in H9c2 cells.

A. Cells were cultured in differentiation conditions (Diff) in absence or in presence of IL-6 up to three days. As evidenced by the haematoxylin/ eosin staining (first lane), IL-6 treated H9c2 cells showed enlarged and elongated phenotype and were often fused to form binucleated cells (arrow heads). Cell dimension were 785±217, 1012±210 and 1817±380 μm^2 *§, in Prol, Diff and Diff+IL6 respectively, *p <.001 relative to Prol, §p<.001 relative to Diff. Immunofluorescence analyses for αMHC (second lane), cTnT (third lane) and Conn43 (last lane) (green fluorescence) evidenced a weak and finely dotted reaction in Prol, that increased in Diff and appeared still more intense in presence of IL-6. Note that fluorescence deeply marked the binucleated cells, where the positivity for αMHC and cTnT was not dotted but appeared organized in oriented microfilaments. Nuclei were counterstained in blue by Dapi. Original magnifications: 40X. **B.** Immunofluorescence analyses for Nkx2.5 and GATA4 (green fluorescence). Nuclei were counterstained in blue by Dapi. Cells were treated up to 30 minutes with IL-6. The fluorescent intensity clearly increased upon the IL-6 exposure, both at cytoplasmic and nuclear levels. Red arrows indicate the positivity in the nuclei. Original magnification: 40X in the panel, 100X in the inset. **Table.** Percentage of cells with nuclear positivity for Nkx 2.5 or GATA4. The values were determined by direct microscopic examination of images captured from five distinct fields per well at 40x and counting nuclear positive cells versus the total number of cells per field.

Data are representative of five different experiments.

Figure 2: IL-6 treatment affects the expression of IL-6R and the cellular localization of gp130 co-receptor in H9c2 cells.

H9c2 cells were cultured in Diff in presence of IL-6 up to three days. **A.** Immunofluorescent analysis for IL-6R and gp130 (green fluorescence). Nuclei were counterstained in blue by Dapi. After 10 and30 min of IL-6 treatment an increase of IL-6R, but not of gp130, signal was evident at cytoplasmic and perinuclear levels (red arrow heads), while the fluorescent intensity observed in 3 days treated cells was comparable to that observed in the proliferating population. Note a gp130 reinforced signal at plasma membrane in 10 min IL-6 treated sample (arrows). Original magnification 40X. Data are representative of five different experiments. **B.** H9c2 were harvested after 10 min, 30 min or 3 days of IL-6 treatment and cell lysates were subjected to Western blot for IL-6R and gp130 detection. IL-6R immunoreactive band was stronger in 10 and 30 min IL-6 treated

samples. Equal amounts of protein (15 μg) were loaded in each line and G6PDH was used as loading control. Data are representative of five different experiments.

Figure 3: IL-6 treatment activates Jak1-STAT3 and PI3K signaling transduction pathways and induces PTEN and PKCζ activation in H9c2 cells.

H9c2 cells were treated with IL-6 up to 30 min in order to analyze the signal transduction pathways. **A.** Cell lysates were subjected to Western blot analysis for the detection of Jak1, STAT3, Erk1/2, PI3K, Akt and PTEN, as total proteins or phosphorylated forms. The switch in Diff medium up to 30 min did not determined modification of the expression and phosphorylation levels of these molecules. When IL-6 was added to the Diff medium, no modification of total amount of Jak1, STAT3, Erk1/2, PI3K, Akt and PTEN was observed, whereas the phosphorylated form (p) of Jak1, STAT3 (Tyr705) and PI3K increased, the pAkt levels decreased, and the no-phosphorylated PTEN levels incremented. Equal amounts of protein (15 μg) were loaded in each line and G6PDH was used as loading control. Data are representative of five different experiments. **B.** Cell lysates were subjected to Western blot analysis for the detection of PKCα, PKCδ, PKCε and PKCζ, as total proteins or phosphorylated forms. The IL-6 addition did not determined modifications in PKCα, PKCδ and PKCε expression and phosphorylation levels in H9c2 cells, whereas a rapid increment of pPKCζ was observed. Equal amounts of protein (15 μg) were loaded in each line and G6PDH was used as loading control. Data are representative of five different experiments. **C.** Immunofluorescence analysis for PKCζ and pPKCζ (green fluorescence). Nuclei were counterstained in blue by Dapi. The anti-PKCζ antibody stained the cytoplasm of both untreated and IL-6 stimulated H9c2 cells. The anti-pPKCζ antibody reacted with the unstimulated cells, but an increase of pPKCζ staining was observed in IL-6 treated samples, with a diffuse positivity that localized in the cytoplasm but also in the nuclei (red arrowheads). Original magnification 40X. Similar results were obtained in five additional experiments.

Figure 4: PI3K inhibition blocks the IL-6 signaling activation of PTEN and PKCζ pathways in H9c2 cells.

H9c2 cells were treated the PI3K inhibitor LY294002 for 1 hr before the IL-6 treatment. After 10 and 30 min cells were harvested and lysates were subjected to Western blot analysis for the detection of PKCζ and Akt, as total proteins and phosphorylated forms, and PTEN as total protein and no phosphorylated form. When PI3K activity was blocked the by LY294002, the addition of IL-6 to the medium did not modify the phosphorylation levels of PKCζ, Akt and PTEN did not modify. Equal amounts of protein (30 μg) were loaded in each line and G6PDH was used as loading control. The graphs on the left represent the ratio of the densitometric values relative to

pPKCζ/PKCζ, pAkt/Akt and no-pPTEN/PTEN in IL-6 treated cells for 10 and 30 min in absence (grey columns) and in presence (white columns) of the PI3K inhibitor LY294002. Values are expressed as percent of ratios measured in the untreated cells (100%). Results are expressed as mean ± SD of five separated experiments. * indicates values statistically different ($P<0.01$) from the control samples (untreated cells), whereas § indicates values statistically different ($P<0.05$) between IL-6 treated samples in presence and in absence of LY294002. Data are representative of five different experiments.

Figure 5: PKCζ inhibition does not influence the IL-6 dependent activation of PI3K, whereas affects the Akt and PTEN pathways in H9c2 cells.

PKCζ activity was inhibited by treating the H9c2 cells with PKCζ pseudosubstrate for 1 hr before the IL-6 addition. Cells were harvested after 10 and 30 min of IL-6 treatment cells and lysates were subjected to Western blot analysis for the detection of PI3K, Akt as total proteins and phosphorylated forms and PTEN, as total protein and no phosphorylated form. In IL-6 treated lysates the immunoreacted band relative to pPI3K increased both in absence and in presence of PKCζ inhibitor, whereas pAkt and no-pPTEN levels did not vary when PKCζ activity was blocked. Equal amounts of protein (25 μg) were loaded in each line and G6PDH was used as loading control. The graphs on the left represent the ratio of the densitometric values relative to pPI3K/PI3K, pAkt/Akt and no-pPTEN/PTEN in IL-6 treated cells in absence (grey columns) and in presence (white columns) of the PKCζ pseudo-substrate. Values are expressed as percent of ratios measured in the untreated cells (100%). Results are expressed as mean ± SD of five separated experiments. * indicates values statistically different ($P<0.01$) from the control samples (untreated cells), whereas § indicates values statistically different ($P<0.01$) between IL-6 treated samples in presence and in absence of PKCζ inhibitor.

Figure 6: Gain of PKCζ function determines morphological modifications, influences the expression of specific myocardial markers and modifies Akt and PTEN phosphorylation levels in H9c2 cells .

Cells were electroporated with caPKCζ or iPKCζ expression vectors and analyzed 36 hr after the transfection. Negative controls were represented by mock or GFP transfected cells.

A. Structures of the two HA-tagged PKCζ mutants used in the study. The caPKCζ construct codifies the catalytic domain of the enzyme. The iPKCζ construct encodes the full-length protein with a point mutation leading to the Lys to Arg substitution at position 368 of the catalytic domain that impairs its kinase activity (Nakashima, 2002). Western blot analysis for the expression of the

PKCζ mutants in transiently transfected in H9c2 cells evidenced that cells produced comparable levels of the ectopic proteins. Cell lysates were resolved by SDS–PAGE, blotted and probed with an anti-HA. Equal amounts of protein (15 μg) were loaded in each line. **B.** Hematoxylin/ eosin staining (top lane) and immunofluorescence for αMHC (middle lane) and cTnT (bottom lane) of caPKCζ and iPKCζ transfected cells. Relative to control, caPKCζ transfected cells were more elongated, often fused in binucleated sincythia (red arrow heads) and expressed higher levels of αMHC and cTnT (green fluorescence). Nuclei were counterstained in blue by Dapi. Note that fluorescence deeply marked the binucleated cells. Transfection with iPKCζ was ineffective on cell morphology and αMHC and cTnT. Original magnifications: 40X. **C.** Immunofluorescence analyses for Nkx2.5 and GATA4 (green fluorescence). Nuclei were counterstained in blue by Dapi. The fluorescent intensity increased in caPKCζ transfected cells, particularly at nuclear levels (red arrows). Original magnification: 100X. **D.** Lysates were subjected to Western blot analysis for the detection of Akt as total proteins and phosphorylated form and PTEN, as total protein and no phosphorylated form. Relative to controls, caPKCζ transfected sample displayed lower pAkt and higher no-pPTEN levels; the transfection with iPKCζ was ineffective on PTEN phosphorylation levels and slightly increased the pAkt. Equal amounts of protein (20 μg) were loaded in each line and G6PDH was used as loading control. The graphs on the left represent the ratio of the densitometric values relative to pAkt/Akt and no-pPTEN/ PTEN in caPKCζ (grey columns) and iPKCζ (white columns) transfected cells. Values are expressed as percent of ratios measured in the untreated cells (100); * indicates values statistically different ($P<0.05$) from the control samples (untreated cells), whereas § indicates values statistically different ($P<0.05$) between caPKCζ and iPKCζ transfected cells.

All data are representative of at least five different experiments.

Figure 7: Schematic representation of the signal pathway model proposed for the IL-6 effects in H9c2 cells. The IL-6 interaction with its receptor complex activates a PI3K - PKCζ cascade. PKCζ determines, directly or indirectly (dotted line) a PTEN dephosphorylation with a consequent inhibition of the Akt activity.