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STAT1 requirement for PKR-induced cell cycle arrest in vascular smooth muscle cells in response to heparin

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ABSTRACT

Interferons (IFNs) are a family of cytokines that exhibit antiviral, antiproliferative, and immunomodulatory properties. PKR (protein kinase, RNA activated) is of central importance in mediating the antiproliferative actions of IFNs. Our research has established that PKR inhibits vascular smooth muscle cell (VSMC) proliferation by regulating G1 to S transition. Many cardiovascular diseases result from complications of atherosclerosis, a chronic and progressive inflammatory condition often characterized by excessive proliferation of VSMC. Thus, an effective method for inhibiting VSMC proliferation is likely to arrest atherosclerosis and restenosis at early stages. Our research establishes that PKR activation in VSMC leads to a G1 arrest brought about by an inhibition of cyclin-dependent kinase 2 (Cdk2) activity by $p27^{kip1}$. In quiescent VSMC, $p27^{kip1}$ levels are high and when stimulated by serum/growth factors, $p27^{kip1}$ levels drop by destabilization of the protein. Under conditions that lead to activation of PKR, there is a marked inhibition of $p27^{kip1}$ down-regulation due to increased stability of $p27^{kip1}$ protein. In order to understand the mechanism of heparin-induced stabilization of $p27^{kip1}$ in VSMC, we examined the involvement of the Signal Transducer and Activator of Transcription-1 (STAT1), which is an important player in mediating antiproliferative effects of IFNs. Our results demonstrate that PKR overexpression in VSMC leads to an increase in $p27^{kip1}$ protein levels and this increase requires the catalytic activity of PKR. PKR activation induced by antiproliferative agent heparin leads to phosphorylation of STAT1 on serine 727, which is essential for the cell cycle block. STAT1 null VSMCs are largely defective in heparin-induced cell cycle arrest and in PKR null cells the STAT1 phosphorylation in response to heparin was absent. These results establish that heparin causes STAT1 phosphorylation on serine 727 via activation of PKR and that this event is required for the G1 arrest in VSMC.

1. Introduction

The proliferation of VSMCs in vessels after endothelial injury contributes to the myointimal plaque (Ross, 1993), which plays an important role in atherosclerosis. In the normal artery, smooth muscle cells remain in a quiescent state and their proliferation is a hallmark of the early stages of the disease (Schwartz et al., 1986). Regulation of VSMC proliferation may therefore help to prevent formation of atherosclerotic plaques or to reduce their size. Heparin is an antiproliferative agent for VSMCs (Aikawa et al., 1993; Kocher et al., 1991; Sjolund et al., 1988; Thyberg et al., 1990) and our previous work demonstrated that heparin treatment of VSMC results in activation of protein kinase PKR by direct binding after internalization of heparin (Patel et al., 2002). Activation of PKR by heparin induces a cell cycle block at G1 to S phase transition (Patel et al., 2002) by inhibition of $p27^{kip1}$ degradation (Fasciano et al., 2005a, 2005b) leading to a rise in cellular $p27^{kip1}$ protein levels and consequent inhibition of cyclin-dependent kinases (CDKs). In PKR null cells, heparin's antiproliferative effects are diminished and the G1 to S phase transition block is impaired (Patel et al., 2002). Inhibition of PKR activity in VSMC by 2-aminopurine, an inhibitor of PKR, also results in a loss of $p27^{kip1}$ stabilization in response to heparin (Fasciano et al., 2005a, 2005b). Thus, PKR is essential for mediating heparin's antiproliferative actions in VSMC.

PKR is an ubiquitously expressed key mediator of the antiviral and antiproliferative actions of interferons (IFNs) (Barber, 2005; Barber et al., 1995; Clemens and Elia, 1997). Although PKR synthesis is induced at the transcriptional level by IFNs (Hovanessian, 1989), its kinase activity stays latent until it binds to its activator. PKR undergoes autophosphorylation and enzymatic activation upon binding to any one of the three activators: double-stranded (ds) RNA (Galabru and Hovanessian, 1987), heparin (Hovanessian and Galabru, 1987), and protein activator PACT (Patel and Sen, 1998), acting as activators in response to unique signals. The dsRNA dependent activation of PKR occurs during viral infections (Katze, 1995), and PACT activates PKR in response to cellular stress (Ito

Abbreviations: IFN, interferon; PKR, protein kinase, RNA-activated; VSMC, vascular smooth muscle cell; Cdk2, cyclin-dependent kinase 2; STAT1, signal transducer and activator of transcription-1; ds, double-stranded; JAK1, janus kinase 1; ATRA, all-trans retinoic acid; FACS, fluorescence assisted cell sorting; eIF2α, eukaryotic initiation factor 2 alpha subunit; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

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et al., 1999; Patel et al., 2000; Peters et al., 2006; Singh et al., 2011). Both dsRNA and PACT bind to PKR through its two evolutionarily conserved dsRNA binding motifs (dsRBMs), which are involved in both dsRNA-binding and protein–protein interactions (Banerjee et al., 1990; Green and Mathews, 1992; Patel and Sen, 1992; St Johnston et al., 1992). On the contrary, heparin binds to PKR via PKR's heparin-binding domains that are present in the carboxy-terminal half and overlap with the catalytic domains (Fasciano et al., 2005a, 2005b). Phosphorylation of the alpha subunit of the protein synthesis initiation factor eIF2 (eIF2α by PKR on serine 51) leads to a global inhibition of protein synthesis (Clemens, 2001).

Generation of mice with a targeted disruption of PKR gene (Kumar et al., 1997) has delineated the cellular functions of PKR in growth regulation, and in pro-inflammatory and apoptotic signaling (Williams, 1995). PKR is a negative regulator of cell proliferation, and an over-expression of PKR inhibits cell proliferation. Expression of catalytically inactive mutants of PKR in NIH 3T3 cells results in a transformed phenotype due to a transdominant inhibitory effect of mutant enzyme on the endogenous PKR (Koromilas et al., 1992; Meurs et al., 1993). Similarly, overexpression of $p58^{IPK}$ (a cellular inhibitor of PKR), and E3L (vaccinia virus encoded inhibitor of PKR) also results in oncogenic transformation of NIH 3T3 cells (Barber et al., 1994; Benkirane et al., 1997; Garcia et al., 2002).

In order to understand the mechanism of heparin-induced stabilization of $p27^{kip1}$ in VSMC, we wanted to examine the involvement of various signaling pathways regulated by PKR, the Signal Transducer and Activator of Transcription-1 (STAT1) being a major player since STAT1 phosphorylation is induced by antiproliferative signals (Kim and Lee, 2007). STAT is a family of cytoplasmic proteins with roles as signal messengers and transcription factors that participates in cellular responses to cytokines and growth factors (Stark and Darnell, 2012). STAT1, the prototypical family member, plays an essential role in innate immunity by protecting the host from infections with viruses and other pathogens. STAT1 is activated in response to IFNs by phosphorylation on tyrosine 701 by Janus kinases (JAKs), which is required for its dimerization, translocation to the nucleus, and binding to DNA (Platanias, 2005). In addition to this, phosphorylation on serine 727 is required for transcriptional responses to IFN (Nguyen et al., 2001; Uddin et al., 2002; Varinou et al., 2003; Wen and Darnell, 1997). STAT1 exhibits tumor-suppressor functions through its ability to promote the immunosurveillance of tumors in mouse models (Wang et al., 2008) and by inhibiting the proliferation of tumor cells in response to IFN-γ through the upregulation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ in cell culture (Chin et al., 1996). STAT1 is required for IFN-γ induced growth arrest and both tyrosine 701 and serine 727 phosphorylations are essential for this cell cycle block (Ramana et al., 2000; Raveh et al., 1996). Ser727/Tyr701-phosphorylated STAT1 plays a key role for the all-trans retinoic acid (ATRA)-induced growth arrest, down-regulation of c-myc and simultaneous up-regulation of $p27^{kip1}$ (Dimberg et al., 2003). Recently it was also demonstrated that STAT1 exhibits a strong antitumor function in Ras-transformed cells and tissues by upregulating $p27^{Kip1}$(Wang et al., 2010). Although STAT1 induces $p27^{Kip1}$ at the transcriptional level (Wang et al., 2008), it also suppresses *Skp2* gene transcription and promotes $p27^{Kip1}$ stabilization in Ras-transformed cells (Wang et al., 2010).

There is some evidence that PKR is essential for inducing serine 727 phosphorylation of STAT1 in response to antiproliferative signals. PKR is essential for IFN-γ induced STAT1 phosphorylation on serine 727, growth arrest, and c-myc down-regulation (Ramana et al., 2000). In this report, we examined the effect of PKR overexpression in VSMC and a possible involvement of STAT1 activity in PKR-induced $p27^{kip1}$ stabilization and G1 arrest. Our results indicated that PKR overexpression causes increase in $p27^{kip1}$ protein levels and this increase requires the catalytic activity of PKR (Patel et al., 2002). Here we show that PKR activation induced by heparin leads to phosphorylation of STAT1 on serine 727, which is essential for the cell cycle block. The heparin-induced cell cycle arrest was largely absent in STAT1 null VSMCs and in PKR null cells the STAT1 phosphorylation in response to heparin was absent. These results strongly suggest that heparin causes STAT1 phosphorylation on serine 727 via activation of PKR and that this event is required for the G1 arrest.

2. Methods and materials

2.1. Generation of stable VSMC lines overexpressing human wt PKR

A10 tTA-IRES-Neo cells were kindly provided by Dr. Robert Weiss (University of California, Davis). These cells were used to generate stable clones that overexpress wt PKR in response to removal of tetracycline from the growth medium. Wt PKR was cloned in pTRE2-puro (Clontech) and stable lines were generated by selecting clones resistant to puromycin at 600 ng/ml concentration. Several clones were characterized and one that showed tight repression under tet ON condition and good induction under tet OFF condition was selected for further experiments.

2.2. STAT1 null VSMCs

The $STAT1^{-/-}$ VSMCs established from $STAT1^{-/-}$ mice were kindly provided by Dr. Inna Dumler (Hannover Medical School, Germany). These cells were cultured in SmGM2 cell culture medium from Clonetics supplemented with 5% FBS. The cells could also be cultured in DMEM supplemented with 10% FBS. There were no measurable differences in growth characteristics under two conditions and either growth medium could be used for their propagation.

2.3. Western blot analysis

The cells were grown in 100-mm plates and were treated as indicated in the figure legends. Cell extracts were prepared in 20 mM Tris–HCl, pH 7.5, 100 mM KCl, 200 mM NaCl, 4 mM MgCl2, 2 mM dithiothreitol, 2% Triton X-100, 40% glycerol, 20 mM sodium fluoride, 20 mM sodium pyrophosphate, 20 mM μ-glycerophosphate, 20 mM sodium molybdate, 0.4 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin. 50–100 μg of total cellular proteins were separated by SDS-PAGE and subjected to western blot analysis using the ECL plus reagents (Amersham Biosciences) according to the manufacturer's directions and following specific antibodies: PKR (R &D Systems, MAB1980, 1:10,000); α-smooth muscle actin (Sigma, A2547 clone 1A4, 1:1000); p27 (Santa Cruz Biotechnology, sc-1641, 1:1000); STAT1 (Santa Cruz, sc-271661, 1:1000); phospho-PKR-Thr451 (Cell Signaling, #3075, 1:1000); and phospho-STAT1-ser727 (Cell Signaling, #9177, 1:1000).

2.4. Statistics

For the analysis of fold differences in STAT1 phosphorylation or $p27^{kip1}$ protein levels between the cell lines, a two-tailed Student's *t* test analysis was performed with equal variance. For the cell cycle analysis similar statistical analysis was performed using a two-tailed Student's *t* test. A value of $P < 0.01$ was considered statistically significant.

2.5. Cell cycle analysis

The VSMCs were cultured and treated as described in the Fig. 3 legend and analyzed by flow cytometry as described (Patel et al., 2002) with the use of a Coulter Flow Cytometer.

3. Results

Our previous research has indicated that heparin-induced PKR activation in VSMC leads to a block in G1 to S phase transition due to stabilization of CDK inhibitor protein $p27^{kip1}$. In addition to PKR