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TNF-α and leptin activate the α-isoform of class II phosphoinositide 3-kinase

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Abstract

The class II PI 3-kinases are known to be activated by growth factors and chemokines but to date there are no reports of cytokine mediated regulation. Further, the intracellular signalling mechanisms regulating the class-II PI 3-kinases are poorly understood. We investigated the effects of the cytokines TNFα and leptin on the activity of the α isoform of the class II PI 3-kinase (PI3K-C2α) and find that these stimulate the enzyme 2-fold and 3-fold, in CHO cells and J774.2 macrophages, respectively. The stimulation by leptin was not accompanied by recruitment of any tyrosine phosphorylated proteins to PI3K-C2α and no shift in electrophoretic mobility was noted. Furthermore, we demonstrate that the actions of both cytokines are blocked by the MEK inhibitor PD98059. These findings indicate that the cytokines activate PI3K-C2α and do so by a mechanism that requires activation of the ERK pathway and thus differs from the mechanism used by insulin to activate the enzyme.

A large body of evidence has accumulated to indicate that phosphoinositide (PI)-3-kinase activity plays a crucial role in regulating a number of cellular processes including cell growth, cell motility, gene expression, and metabolism [1–3]. PI 3-kinases are a ubiquitously expressed enzyme family that are able to phosphorylate membrane inositol lipids at the third position of the inositol ring. PI 3-kinase was originally purified and cloned as a heterodimeric complex consisting of a 110 kDa catalytic subunit and an 85 kDa regulatory adapter subunit. This form of PI 3-kinase has been widely studied with three homologous genes for the catalytic subunit and three for the adapter subunit having been identified. These have been termed the class I PI 3-kinases [1–3]. The class II PI 3-kinases represent a novel but poorly characterised group of PI 3-kinases characterised by the presence of a C2 domain and a Phox homology domain (PX) at the extreme C terminus. Three mammalian isoforms of the class II PI 3-kinases (PI3K-C2) have been identified, with PI3K-C2α [4–6] and PI3K-C2β [7,8] being widely expressed and PI3K-C2γ restricted to liver [9,10]. The in vitro substrate specificity of all class II PI 3-kinases is similar, in that they prefer PI as a substrate but they also phosphorylate PI 4-phosphate to a lesser extent, and can phosphorylate PI (4,5)P2 at low levels under certain conditions [5,11]. The role of class II PI 3-kinases in the cell and how they might be involved in signalling cascades are poorly understood, but, given the importance of PI 3-kinases in regulating cellular function, it is clearly of interest to investigate how they are regulated and by what mechanism. One thing that is clear is that the catalytic activity of the class-II PI 3-kinases can be increased by a number of stimuli. For example, the chemokine, monocyte chemotactic peptide-1 (MCP-1), induces a transient activation of PI3K-C2α via a $G_{\alpha i}$-linked mechanism in monocytes [12]. The activity of PI3K-C2α can also be increased by growth factors. Epidermal growth factor (EGF) activates via a mechanism that involves recruitment to the EGF receptor[13,14] and insulin activates by a mechanism that involves recruitment to a 160 kDa tyrosine phosphorylated molecule [15,16]. Both also cause a shift in the electrophoretic mobility of PI3K-C2α which may have functional implications. There are no reports of how cytokines affect PI3K-C2α activity.

Leptin and tumour necrosis factor (TNF) α are cytokines [17,18] with elevated levels of both being found

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in obese individuals [19–21]. Leptin and TNFα exhibit different mechanisms of signal transduction. Leptin signals primarily through the long form of the receptor (ObRb) which has signalling capabilities of IL-6 type cytokine receptors [22]. This class of cytokines induces homodimerisation of their cognate receptors, resulting in activation of associated Janus kinases (JAK) and subsequent signal transducer and activator of transcription 3 (STAT3) activation [23]. We have recently demonstrated that ObRb is expressed to high levels in macrophages [24] and is capable of activating the class I PI 3-kinases [24,25]. TNFα exerts its effects by its binding to TNF receptor (TNFR)1 and TNFR2, expressed on virtually all types of cells [26,27]. TNFR1 has been found to initiate the majority of TNF biological activities. The binding of TNF to TNFR1 leads to recruitment of TRADD (TNFR associated death domain) and various other adapter proteins, responsible for initiating signalling events leading to the activation of two major transcription factors, nuclear factor κB (NF-κB) and c-jun. Other pathways are also activated in response to TNFα. For example, it has recently been shown that TNFα induces expression of Foxc2 (winged helix/fork head gene) via a class I PI3K and ERK 1/2 dependent mechanism in adipocytes [28]. To date, there have been no studies of regulation of class-II PI 3-kinases by either leptin or TNFα, or indeed any other cytokine. We find that both are capable of rapidly increasing PI3K-C2α and, unlike the activation by insulin, these effects are blocked by the MEK inhibitor PD98059.

Materials and methods

Antibodies and reagents. All chemical reagents were from Sigma unless otherwise stated. Radiochemicals were obtained from Amersham. PI 3-kinase reagents were as described previously [15]. Antibodies to ERK, p38 MAPK, and PKC antibodies were obtained from New England Biolabs. PI3K-C2α antibody was a gift of the Waterfield laboratory (Ludwig Institute London). PD98059 MEK inhibitor was obtained from Calbiochem. Recombinant murine leptin was obtained from Biogenesis. Recombinant murine TNFα was obtained from R&D systems.

Cell culture. The murine macrophage cell line J774.2 was grown in RPMI medium (2 g glucose/l), supplemented with 10% FBS and 1% antibiotic–antimycotic. J774.2 macrophages were serum starved in RPMI media containing 0.2% BSA (fatty acid free) overnight as previously described [15]. CHO-IR cells were obtained from Prof. K. Siddle (University of Cambridge). The CHO-IR cell line was grown in HAMS-F12 medium supplemented with 10% FBS and 1% antibiotic–antimycotic. CHO-IR cells were serum-starved in HAMS-F12 media without FBS overnight.

Cell lysis, immunoprecipitation, and immunoblotting. Cells were treated as indicated, then washed once in phosphate-buffered saline, and lysed in a buffer containing 10 mM Tris–HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM NaF, and 1% (v/v) Triton X-100 supplemented with 2 μg/ml aprotinin, 1 mM pepstatin, 1 μg/ml leupeptin, 2 mM PMSF, and 1 mM sodium orthovanadate. For Western blotting cell lysates (50 μg) were resolved by SDS–PAGE on an 8% gel transferred to polyvinylidene difluoride, and incubated with polyclonal antibodies to PI3K-C2α, phospho, and total ERK. Western blots were visualised with enhanced chemiluminescence (ECL) and analysed using a Fuji Las1000 system.

PI 3-kinase activity assay. PI 3-kinase assays were performed on the PI 3KC2α immunoprecipitates with phosphatidylinositol as a substrate using the methods previously described [15]. TLC plates were analysed using Fuji FLA-2000 phosphoimager.

Statistical comparisons. Differences between data were analysed using the Student's t test (means ± SEM).

Results

We have previously shown that leptin is maximally effective at 2 nM in J774.2 macrophages [24] and we find here that this concentration leptin significantly increased PI3K-C2α activity in these cells (Fig. 1). This activation was maximal after 15–20 min of leptin stimulation, where an approximate 4-fold increase in PI3K-C2α activity was observed. Leptin-stimulated PI3K-C2α activity was reduced to basal levels by pre-treatment of the cells with the MEK inhibitor PD98059 (Fig. 2), using concentrations which we have previously shown to block the activation of ERKs in these cells [29]. In contrast with insulin [15], stimulation with leptin did not cause recruitment of any tyrosine phosphorylated proteins into PI3K-C2α immunoprecipitates and no shift in electrophoretic mobility of PI3K-C2α could be detected after leptin stimulation (data no shown).

Stimulation of CHO-IR cells with 25 ng/ml TNFα also significantly increased PI3K-C2α activity (Fig. 3A).

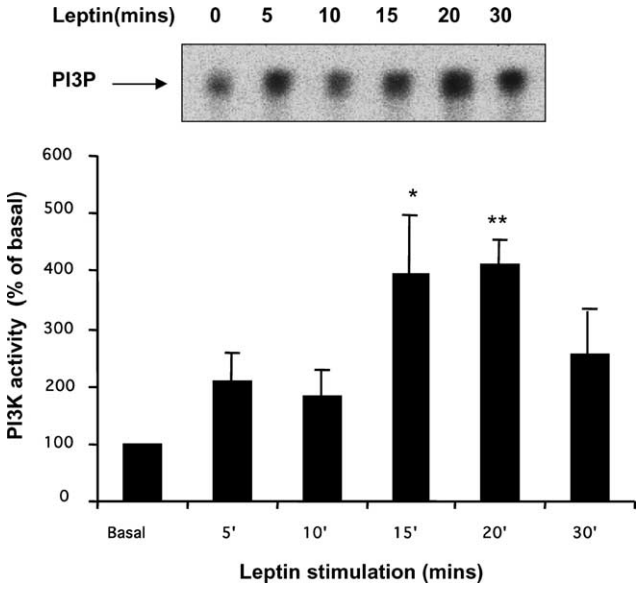

Fig. 1. Leptin stimulation activates PI3K-C2α activity in macrophages. Macrophage cells were serum-starved overnight and then stimulated with 2 nM leptin for indicated times. Cells were lysed and immunoprecipitated using PI3K-C2α specific antisera. PI 3-kinase assays were performed on these immunoprecipitates as described under "Materials and methods." Results are means of four experiments carried out in quadruplet. Results are expressed as percentage of basal (±SEM). *$p < 0.05$ vs basal and **$p = 0.01$ vs basal.

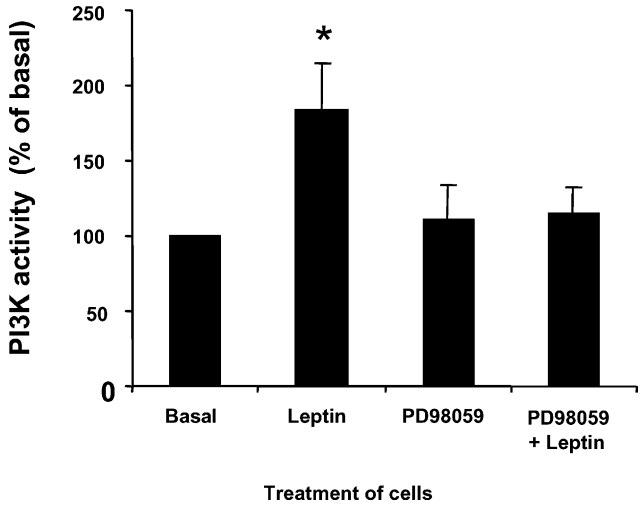

Fig. 2. PD98059 blocks leptin stimulation of PI3K-C2α activity in macrophages. Macrophage cells were serum-starved overnight and then incubated with 50 μM PD98059 prior to addition of 2 nM leptin for 20 min. Cells were lysed and immunoprecipitated using PI3K-C2α specific antisera. PI 3-kinase assays were performed on these immunoprecipitates, as described under "Materials and methods." Results are a representative of three independent experiments carried out in duplicate. Results are expressed as percentage of basal (±SEM).

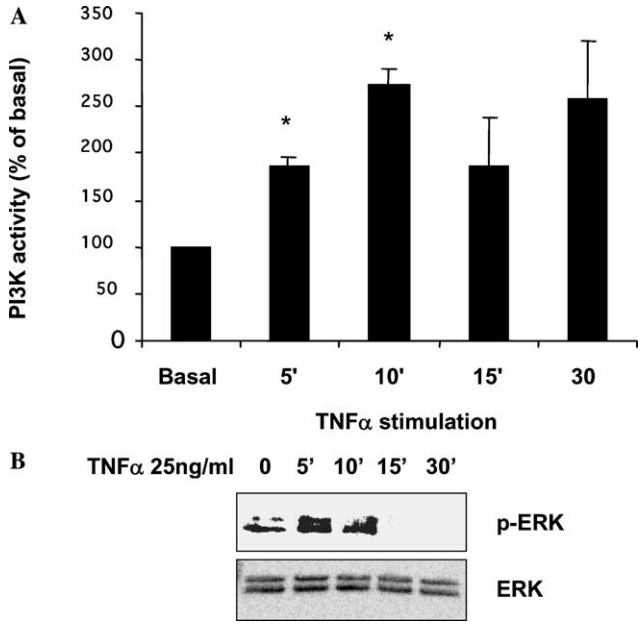

Fig. 3. TNFα stimulation causes phosphorylation of MAP kinases and activates PI3K-C2α activity in CHO-IR cells. CHO-IR cells were serum-starved overnight and then stimulated with 25 ng/ml recombinant TNFα for indicated times. Cells were lysed and immunoprecipitated using PI3K-C2α specific antisera. PI 3-kinase assays were performed on these immunoprecipitates as described under "Materials and methods" (A). Cell lysates (50 μg) were subjected to SDS–PAGE, as described under "Materials and methods" and Western blotted with phospho and total ERK and p38 MAPK specific antisera (B). Results are a representative of three independent experiments carried out in duplicate. Results are expressed as percentage of basal (±SEM).

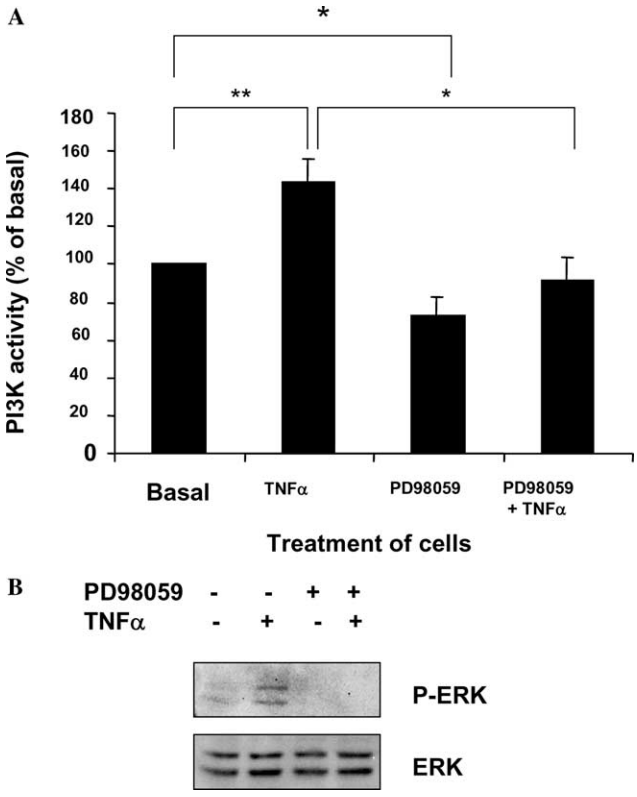

Fig. 4. TNFα stimulation of PI3K-C2α activity is ERK dependent in CHO-IR cells. CHO-IR cells were serum-starved overnight and then stimulated with 50 μM PD98059 prior to 25 ng/ml recombinant TNFα for 10 min. Cells were lysed and immunoprecipitated using PI3K-C2α specific antisera. PI 3-kinase assays were performed on these immunoprecipitates as described under "Materials and methods" (A). Cell lysates (50 μg) were subjected to SDS–PAGE, as described under "Materials and methods" and Western blotted with phospho and total ERK specific antisera (B). Results are means of four experiments carried out in duplicate. Results are expressed as percentage of basal (±SEM). *$p < 0.05$ vs basal and **$p = 0.01$ vs basal.

This activation was maximal after 10 min of stimulation, where an approximate 2–3-fold increase in PI3K-C2α activity was observed. Stimulation with TNFα for approximately 5–10 min resulted in transient phosphorylation of both ERK1 and ERK2 (Fig. 3B). Further, we found that the TNFα-induced PI3K-C2α activity was reduced to basal levels with PD98059 pre-treatment (Fig. 4A). Western blotting with phospho-specific ERK 1/2 antibodies confirmed that ERK activation was blocked by pre-incubation with PD98059 inhibitor (Fig. 4B).

Discussion

A great deal of evidence has emerged showing PI 3-kinase activity as being an essential component of a wide range of cellular signalling pathways. However, very little is known about the novel Class II PI 3-kinases and surprisingly it is currently not known if this form of PI

3-kinase is activated by cytokines. Leptin and TNFα are two cytokines which act through receptors known to have different signalling mechanisms [30] but both have previously been shown to activate class I PI 3-kinase activity [28,31–33]. Here, we show that PI3K-C2α is activated by both these cytokines.

Very little is known about the intracellular signalling pathways involved in the activation of PI3K-C2α. Leptin caused maximal PI3K-C2α activation after 20 min, which correlates well with previously reported data on leptin signalling in J774.2 macrophages, where activation of ObRb, JAK-2, STAT3, and ERK all reach maximal levels after 15–20 min of leptin stimulation [24,29] and we find that TNFα also causes a rapid but transient activation of ERK. In an effort to gain insight into the signalling pathways involved in regulating PI3K-C2α we used the MEK inhibitor, PD98059, to dissect the pathways involved. Both the leptin and the TNFα induced stimulation of PI3K-C2α activity were blocked by PD98059, suggesting that activation is ERK-dependent in response to these cytokines. As far as we are aware there is no evidence available to indicate that ERK is involved in regulating the class-I PI 3-kinases.

In contrast we find that, despite the fact that insulin activates ERK, insulin stimulation of PI3K-C2α activity is not blocked by PD98059 in CHO-IR cells (CK and PRS manuscript in preparation). This is further supported by the finding that, unlike insulin [15], leptin does not induce a bandshift in or recruitment of PI3K-C2α into complexes with tyrosine phosphorylated molecules. This indicates that cytokines activate PI3K-C2α by a mechanism that is distinct from that used by insulin and shows that there are at least two distinct pathways capable of activating class-II PI 3-kinases.

In summary, as it is known that PI 3-kinase activity plays an important role in cytokine signalling our findings indicate that the class-II PI 3-kinases could make an important contribution to this process. Further, they provide the first evidence that ERK could be involved in the mechanism activating a PI 3-kinase.

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