The activation of p38 MAPK by the β-adrenergic agonist isoproterenol in rat epididymal fat cells

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Abstract Here we report that the β-adrenergic agonist isoproterenol increases the activity of the stress-activated kinase p38 MAPK over 10-fold in freshly isolated rat epididymal fat cells. Stimulation of the kinase was rapid, sustained for at least 60 min and sensitive to the specific p38 MAPK inhibitor, SB 203580. Half-maximal stimulation of p38 MAPK by isoproterenol occurred at 13 nM isoproterenol. The cell permeable cyclic AMP analogue, chlorophenylthio-cyclic AMP increased p38 MAPK activity to a similar extent to isoproterenol, suggesting that the effect of the β-adrenergic agonist is mediated via increases in the activity of cyclic-AMP dependent protein kinase. Although it had little or no effect on the activity of c-Jun N-terminal kinase, isoproterenol and a number of other treatments which activated p38 MAPK were found to stimulate AMP-activated protein kinase in fat cells. Activation of AMPK and p38 MAPK were not, however, found to be directly linked.

Key words: p38 MAPK; Isoproterenol; Adipocyte; AMP-activated protein kinase; Cyclic AMP

1. Introduction

Mammalian cells respond to environmental stresses such as UV irradiation and osmotic and heat shock by activating protein kinase cascades that give rise to stimulation of the c-Jun N-terminal kinases (JNK/SAPK1s) and p38 mitogen activated protein kinase (MAPK) (also known as SAPK2 and RK). These stress activated protein kinases also respond to inflammatory cytokines such as interleukin-1 and tumour necrosis factor-α (for review see [1]). Activation of JNK family members gives rise to increased transcription via direct phosphorylation of transcription factors such as c-Jun and ATF2 (see [2]). While p38 MAPK also phosphorylates a number of transcription factors including CHOP [3] and ATF2 [4], it has also been shown to phosphorylate and activate the kinases MAPKAP-K2 [5–7], MAPKAP-K3 [8] and MAPK interacting kinase (Mnk1) [9]. Increases in MAPKAP-K2 activity give rise to phosphorylation of cyclic AMP-response-element binding protein (CREB) [7,10] and heat shock protein 27 (HSP27) [10,11], while Mnk1 has been shown to phosphorylate the eukaryotic initiation factor 4E [9]. A number of other substrates for p38 MAPK have also been inferred by use of two specific p38 MAPK inhibitors, SB 203580 and SB 202190 (see [2]).

Recently p38 MAPK has been shown to be activated by agonists signalling through a number of different G protein-coupled receptors, including the β-adrenergic G_s-coupled receptor in human embryonic kidney 293 cells [12]. β-Adrenergic agonists were classically thought to bring about their effects via activation of adenylate cyclase by $G\alpha_s$, and hence by increases in the intracellular levels of cyclic AMP. More recently, much work has focussed on the additional role of $G\beta\gamma$ subunits in signalling through heterotrimeric G proteins (see [13] for a review). The mechanism by which the β-adrenergic agonist isoproterenol brings about its metabolic effects in fat cells is thought to be largely through $G\alpha_s$ activation of adenyl cyclase, although we have recently reported that it stimulates the activity of protein kinase B via a cAMP-independent mechanism [14]. In order to further investigate the signalling pathways activated by isoproterenol in these cells we have determined its effect on the stress-activated protein kinases.

In this paper we demonstrate that isoproterenol stimulates the activity of p38 MAPK in fat cells, and that this activation appears to be brought about via increases in cyclic AMP. Under the conditions used here, we have also shown that isoproterenol stimulates the AMP-activated protein kinase (AMPK), a kinase that is activated in response to metabolic stress. The effect of isoproterenol on the activity of p38 MAPK, however, occurs independently of any changes in AMPK activity.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies against p38 MAPK and JNK, and the substrate proteins GST-Jun and GST-ATF2 [4] were kindly donated by Dr Martin Dickens, University of Leicester, UK. The sheep polyclonal serum raised against AMPK (reactive against both the α1 and α2 isoforms of the catalytic subunit of AMPK) was the generous gift of Dr David Carling, Royal Postgraduate Medical School, Hammersmith Hospital, London, UK. All other materials were as in [14].

2.2. Preparation and incubation of epididymal fat cells

Adipocytes were isolated from the epididymal fat pads of Wistar rats (175–200 g) and incubated as described previously [15]. Cells (150–250 mg dry cell weight) were extracted in 1 ml of ice cold 50 mM HEPES (pH 7.6), 0.2 mM EDTA, 2.2 mM EGTA, 1 mM dithiothreitol, 100 mM KCl, 10% glycerol, 1% Triton X-100, 1 μM microcystin and 1 μg/ml each of pepstatin, leupeptin and antipain. Extracts were centrifuged at $10\,000 \times g$ for 10 min at 4°C prior to use, and the infranatant taken for the measurement of protein kinase activity.

2.3. Assays of p38 MAPK and JNK

p38 MAPK was immunoprecipitated from cell extracts using a 1:200 dilution of anti-p38 MAPK serum plus 5 mg protein A-Sephar-

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Abbreviations: AICAR, 5-amino-4-imidazolecarboxamide ribonucleoside; AMPK, AMP-activated protein kinase; ATF2, activating transcription factor 2; cpt-cAMP, chlorophenylthio-cyclic AMP; CREB, cyclic AMP response element binding protein; GST, glutathione *S*-transferase; HSP27, heat shock protein 27; JNK, c-Jun N-terminal kinase; p38 MAPK, p38 mitogen activated protein kinase; MAPKAP-K, mitogen activated protein kinase activated protein kinase

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ose. Immunoprecipitates were washed twice and then resuspended in 10 μl assay buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol, 200 μM cyclic AMP-dependent protein kinase inhibitor peptide IP20). The activity of p38 MAPK in immunoprecipitates was assessed using GST-ATF2 (12.5 μg) as a substrate for 15 min at 30°C in the presence of 2 mM $MgCl_2$ and [γ-^{32}P]ATP (100 μM, 200–500 cpm/pmol). JNK activity was similarly assessed in anti-JNK immunoprecipitates using GST-Jun as a substrate. Phosphorylated GST-ATF2 and GST-Jun were run on SDS-PAGE and the degree of phosphorylation was quantified after radioautography by densitometric scanning.

2.4. AMPK assays

For the determination of AMPK activity, fat cells were snap frozen in liquid nitrogen prior to extraction in ice-cold 0.25 M mannitol, 50 mM Tris-HCl (pH 7.2), 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM benzamidine, 1 mM dithiothreitol and 1 μg/ml pepstatin, leupeptin and antipain. Extracts were prepared as above and AMPK immunoprecipitated using 2 μl anti-AMPK antiserum plus 10 μl 50% protein G slurry per ml of cell extract. Immunoprecipitates were washed three times with, and finally resuspended in, AMPK assay buffer (40 mM HEPES (pH 7.0), 80 mM NaCl, 8% (v/v) glycerol, 0.8 mM EDTA, 2 mM $MgCl_2$, 200 μM IP20, 1 mM dithiothreitol). Kinase activity was measured in the presence of [γ-^{32}P]ATP (100 μM, 500–1000 cpm/pmol), 200 μM AMP and 200 μM SAMS peptide for 15 min at 30°C [16].

3. Results

To characterise the response to stress in rat epididymal fat cells the activities of both p38 MAPK and JNK were assessed under a variety of conditions (Fig. 1). The activity of p38 MAPK was increased 10.68 ± 0.84 fold ($n = 5$ separate cell preparations) by the β-adrenergic agonist isoproterenol. Similar levels of activation were also seen with the β_3-adrenergic receptor-specific agonist BRL 37344 and adrenalin (results not shown). The cytokine-suppressive anti-inflammatory drug SB 203580, a specific p38 MAPK inhibitor [2], largely ablated the effect of isoproterenol (Fig. 1A), indicating that the SB

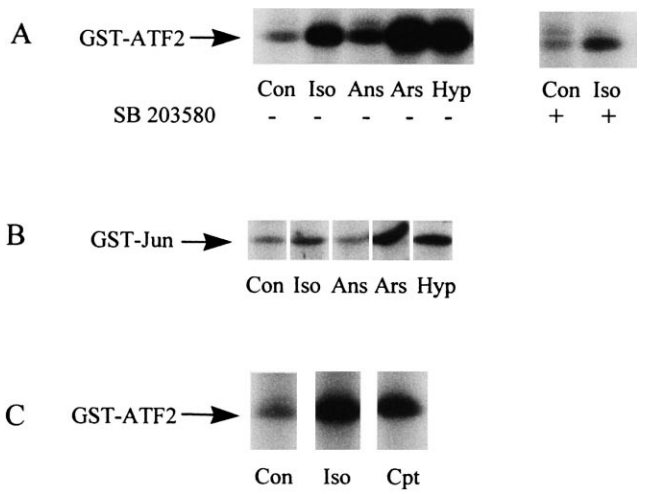

Fig. 1. Activation of p38 MAPK and JNK in adipocytes. Adipocytes were incubated with isoproterenol (Iso, 1 μM), anisomysin (Ans, 10 μg/ml), arsenite (Ars, 5 mM), sorbitol (Hyp, 0.5 M), cpt-cAMP (Cpt, 5 mM) or with no additions (Con) for 10 min. Where indicated, cells were preincubated for 30 min with 5.3 μM SB 203580. The activities of p38 MAPK (A and C) and Jnk (B) were assessed in the same cell extracts as described in Section 2, and phosphorylated GST-ATF2 and GST-Jun were visualised by radioautography. Results shown are representative of at least 3 separate experiments.

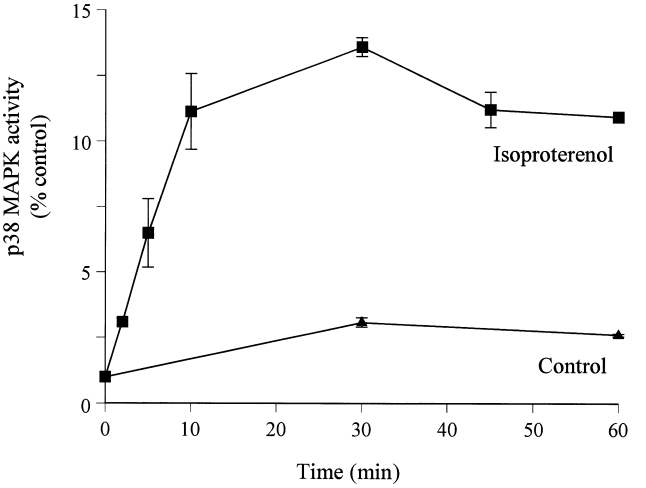

Fig. 2. Time course for activation of p38 MAPK by isoproterenol. Adipocytes were incubated for the times indicated in the presence of 1 μM isoproterenol. The activity of p38 MAPK was quantified as described in Section 2. Results are expressed as percentage of control, unstimulated p38 MAPK activity at time 0 and are means ± S.E.M. of three separate cell preparations.

203580-sensitive α- and β-isoforms of p38 MAPK may be the predominant isoforms affected by isoproterenol in fat cells. Arsenite and hyperosmolarity also potently activated p38 MAPK. Some degree of activation of the kinase was also seen in response to the protein synthesis inhibitor anisomycin. Isoproterenol had little or no effect on the other main stress-activated kinase, JNK, although this was strongly activated by both arsenite and hyperosmolarity (Fig. 1B). Anisomycin had no effect on JNK activity.

In order to investigate the mechanism by which isoproterenol increased the activity of p38 MAPK, the effect of the cell permeable, non-metabolisable cyclic AMP analogue cpt-cAMP was measured. Cpt-cAMP activated p38 MAPK 4.04 ± 0.64-fold ($n = 4$ separate cell preparations) (Fig. 1C), indicating that increased activity of cAMP-dependent protein kinase may underlie the effect of β-adrenergic agonists on p38 MAPK. Activation of phosphatidylinositol 3-kinase does not seem to be involved in the effect of isoproterenol as pretreatment with the inhibitor wortmannin did not inhibit the stimulation of p38 MAPK (data not shown). Insulin, which has no significant effect on p38 MAPK on its own, reversed the stimulation of p38 MAPK caused by low doses (20 nM) of isoproterenol (results not shown).

The time course for the activation of p38 MAPK by isoproterenol (Fig. 2) showed that activation of the kinase peaked after 10–20 min incubation with the agonist, and was sustained for at least 60 min. Over the times studied, the activity of p38 MAPK did not alter significantly in untreated control cells. The dose response curve for the effect of isoproterenol (Fig. 3) shows that half maximal activation of p38 MAPK occurred at 13 ± 3 nM isoproterenol ($n = 3$ separate cell preparations). This EC_{50} for the activation of p38 MAPK was similar to that for the stimulatory effects of isoproterenol on cyclic AMP-dependent protein kinase [17], lipolysis [17] and glycogen breakdown [18].

In addition to studying the effect of isoproterenol on kinases that are involved in the response of cells to environmental stress, we have also determined its effect on a kinase

that is acutely sensitive to metabolic stress, namely the AMP-dependent protein kinase. Under the conditions used to incubate the adipocytes, isoproterenol increased the activity of AMPK activity assayed in the presence of saturating concentrations of AMP (200 μM) over 2.5-fold (Fig. 4). It is therefore likely that the activation seen is a result of phosphorylation and activation of the AMPK in the cells prior to extraction. Fig. 4 also shows that both hyperosmotic shock and arsenite stimulated the AMPK approximately 4-fold in fat cells. Insulin had no effect on the activity of the kinase. Treatment of adipocytes with the cell permeable compound 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) has previously been shown to specifically increase the activity of AMPK [19,20]. AICAR is phosphorylated within the cells to produce AICAR monophosphate (ZMP), which mimics the effect of AMP on AMPK. ZMP activates AMPK both allosterically, and by promoting its phosphorylation by the AMPK kinase [20,21]. Incubating adipocytes with AICAR had no effect on the activity of p38 MAPK, indicating that the stimulation of AMPK was not responsible for the observed increases in p38 MAPK activity seen with isoproterenol, hyperosmotic shock or arsenite (Fig. 4, insert).

4. Discussion

This study shows that isoproterenol increases the activity of the stress-activated protein kinase p38 MAPK in rat fat cells. A number of other reports have also implicated G protein-coupled receptor agonists in the control of p38 MAPK, including f-Met-Leu-Phe in neutrophils [22], thrombin in platelets [23,24], and α-adrenergic agonists in myocardial cells [25,26]. Similarly, Yamauchi et al. [12] have reported that activation of p38 MAPK can also occur in human embryonic kidney 293 cells in response to stimulation of G_s-coupled β-adrenergic receptors, as well as via $G_{q/11}$-coupled m1 and G_i-coupled m2 muscarinic acetylcholine receptors. Their findings, however, indicated that signalling may occur through Gβγ subunits. Isoproterenol has been reported to increase the activity of p38 MAPK in regenerating rat liver, and here the use of a cAMP-dependent protein kinase inhibitor argued against a role for cAMP in this process [27]. In contrast, our present data indicate that p38 MAPK can be activated in fat cells through increases in the intracellular concentration of cAMP, and thus probably through changes in the activity of cAMP-dependent protein kinase.

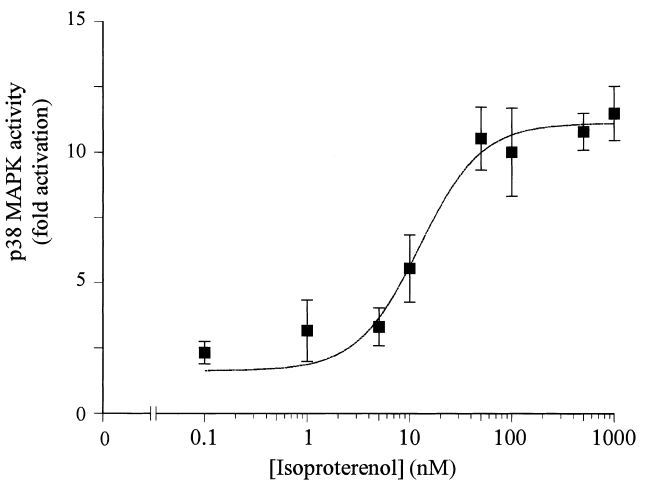

Fig. 3. Dose response for the effect of isoproterenol on p38 MAPK activity. Adipocytes were incubated for 10 min in the presence of the indicated concentrations of isoproterenol. Results are expressed as percentage of the p38 MAPK activity in the absence of isoproterenol and are means ± S.E.M. of three separate cell preparations.

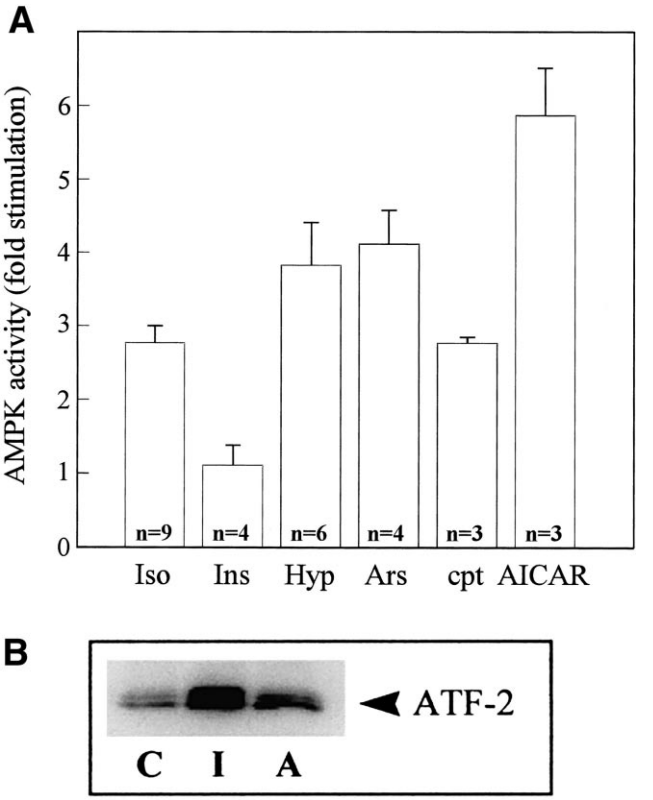

Fig. 4. Effect of isoproterenol on AMPK. Adipocytes were incubated for 10 min in the presence of 1 μM isoproterenol (Iso), 83 nM insulin (Ins), 0.5 M sorbitol (Hyp), 0.5 mM arsenite (Ars), 2 mM cpt-cAMP (cpt) or 2 mM AICAR prior to snap freezing. A: The activity of AMPK was determined as outlined in Section 2 in the presence of 200 μM AMP. Results shown are means ± S.E.M. of the number of separate cell preparations shown. B: Cells were incubated for 10 min in the presence of 1 μM isoproterenol (I), 2 mM AICAR (A) or no further additions (C) prior to extraction and measurement of p38 MAPK activity as described in Fig. 1.

In fat cells the effect of isoproterenol on MAPK signalling pathways appears to be specific for p38 MAPK, with neither the JNK (this paper), nor the classical ERK signalling pathways being activated by β-adrenergic agonists [15]. Indeed, activation of cAMP-dependent protein kinase through $G\alpha_s$-mediated signalling has been shown to suppress activation of ERK1/2 in this cell type [28].

We have previously shown that isoproterenol increases the activity of PKB in adipocytes in a wortmannin-insensitive and cAMP-independent manner [14], and the present studies were initiated in an attempt to define potential signalling pathways involved in this effect. Protein kinase B has been shown to be activated by cellular stress in other cell types [29]. However, the involvement of cAMP in the activation of p38 MAPK suggests that activation of this stress kinase does not underlie the stimulation of protein kinase B by isoproterenol. In addition, we have been unable to show activation of protein kinase B upon treatment of fat cells with other agents that activate p38 MAPK, such as arsenite and hyperosmotic shock (unpublished observations).

Our results also show that isoproterenol gives rise to a stimulation of AMPK in fat cells. This unexpected finding may be a result of ATP depletion that possibly occurs as a result of stimulation of lipolysis by the β-adrenergic agonist [30,31]. Direct effects of isoproterenol, or related agonists, on AMPK activity have not previously been reported. Treatment of fat cells with AICAR has been shown to alter lipid metabolism, with AMPK activation inhibiting both lipogenesis and isoproterenol stimulated lipolysis [19,20]. The effects of AMPK on lipolysis are thought to be brought about through phosphorylation of Ser-565 of hormone-sensitive lipase, with phosphorylation at this site preventing subsequent phosphorylation and activation of the lipase by cAMP-dependent protein kinase [32]. Our current finding that isoproterenol stimulates AMPK may represent a negative-feedback mechanism to control lipolysis in response to β-adrenergic agonists. It may also be involved in the inhibition of acetyl-CoA carboxylase activity and hence lipogenesis. Isoproterenol was found to inactivate acetyl-CoA carboxylase in fat cells primarily by increasing the phosphorylation of Ser-79, the site phosphorylated by the AMPK, rather than by direct phosphorylation of enzyme by cAMP-dependent protein kinase [33].

The activation of p38 MAPK by β-adrenergic agonists in fat cells represents a novel mechanism for the regulation of the p38 MAPK stress pathway, but the full physiological significance remains to be established. At present we cannot eliminate the possibility that isoproterenol activates p38 MAPK through a decrease in cellular ATP content. However, it is clear that the activation of p38 MAPK is not a direct result of AMPK activation as AICAR, a potent activator of AMPK, is without effect on p38 MAPK.

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