RESEARCH COMMUNICATION

Angiotensin II induces tyrosine phosphorylation of insulin receptor substrate 1 and its association with phosphatidylinositol 3-kinase in rat heart

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We have investigated whether angiotensin II (AII) is able to induce insulin receptor substrate 1 (IRS-1) phosphorylation and its association with phosphatidylinositol 3-kinase (PI 3-kinase) in the rat heart *in vivo*. The phosphorylation state of IRS-1 following infusion of insulin or AII via the vena cava was assessed after immunoprecipitation with an anti-peptide antibody to IRS-1 followed by immunoblotting with an anti-phosphotyrosine antibody and an anti-PI 3-kinase antibody. Densitometry indicated a 5.6 ± 1.3-fold increase in IRS-1 phosphorylation after stimulation with AII and a 12.8 ± 3.1-fold increase after insulin. The effect was maximal at an AII concentration of 10^{-8} M and ocurred 1 min after infusion. There was also a 6.1 ± 1.2-fold increase in IRS-1-associated PI 3-kinase in response to AII. In the isolated perfused heart the result was similar, showing a direct effect of AII on this pathway. When the animals were pretreated for 1 h with DuP 753, a non-peptide AII-receptor 1 (AT1 receptor) antagonist, there was a marked reduction in the AII-induced tyrosine phosphorylation of IRS-1, suggesting that phosphorylation is initially mediated by the AT1 receptor. We conclude that AII stimulates tyrosine phosphorylation of IRS-1 and its association with PI 3-kinase. This pathway thus represents an additional signalling mechanism stimulated by AII in the rat heart *in vivo*.

INTRODUCTION

Several lines of evidence indicate that angiotensin II (AII) acts as a cellular growth factor and is involved in the regulation of growth in different tissues in response to a variety of physiological and pathological processes [1]. In addition, AII has been implicated in the rapid developmental growth of the left ventricle during the neonatal period and in the development of pressure-overload left-ventricular hypertrophy [2–4].

AII, acting via the AII-receptor 1 (AT1 receptor), has been reported to stimulate phosphatidylinositol-specific phospholipase C activity through the activation of at least one closely coupled G-protein [1,5]. It has also been demonstrated that AII induces the expression of growth-associated early (*jun*, *fos*) and intermediate (*myc*) proto-oncogenes [6–9]. These data suggest that AII induces proximal and distal signalling events which ultimately lead to cardiac cellular growth. The phosphorylation of tyrosine residues is an early and critical event in transmembrane pathways which promote cell growth [10–12]. Recent studies using various cell types have demonstrated that AII stimulates the tyrosine phosphorylation of several different substrates [13–16].

Insulin and insulin-like growth factor-1 (IGF-1), which act via a cascade involving tyrosine phosphorylation [17], have been clearly implicated in cellular growth [18]. A major substrate for insulin and IGF-1 receptor tyrosine kinase is the src homology 2 ('SH2')-docking protein insulin receptor substrate 1 (IRS-1), which associates with insulin and IGF-1 receptors and links them to downstream signalling pathways [17]. Phosphorylated IRS-1 binds and activates phosphatidylinositol 3-kinase (PI 3-kinase), and these early steps in insulin and IGF-1 action are important for the growth-promoting effects of the two hormones [17–19]. IRS-1 was recently demonstrated to be a substrate for growth hormone (GH) [20], which is also able to induce cell growth. Since AII is a growth factor and can induce the tyrosine phosphorylation of many proteins (including $p125^{FAK}$, $p46^{SHC}$, $p56^{SHC}$ and $p44^{MAPK}$) [16], we have evaluated the ability of AII to stimulate the tyrosine phosphorylation of IRS-1 and the association of phosphorylated IRS-1 with PI 3-kinase in the rat heart *in vivo*. Our data indicate that, following stimulation by AII there is an increase in the tyrosine phosphorylation levels of IRS-1, as well as in the association of IRS-1 with PI 3-kinase, in the heart of intact animals.

MATERIALS AND METHODS

Materials

Reagents for SDS/PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA, U.S.A.). Hepes, PMSF, aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, AII and BSA (fraction V) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein A–Sepharose 6MB was from Pharmacia (Uppsala, Sweden), ^{125}I-Protein A was from ICN Biomedicals (Costa Mesa, CA, U.S.A.) and nitrocellullose paper (BA85; 0.2 mm) was from Schleicher und Schuell. Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly. Anti-IRS-1 antibodies were raised in rabbits using a synthetic peptide derived from the 15 amino acids of the C-terminus of rat IRS-1 and has been previously characterized [21]. Anti-(PI 3-kinase) antiserum and anti-phosphotyrosine monoclonal antibody were from UBI (Lake Placid, NY, U.S.A.).

Methods

Animals and tissue extracts

Male rats (150–180 g) were allowed free access to standard

Abbreviations used: AII, angiotensin II; IRS-1, insulin receptor substrate 1; PI 3-kinase, phosphatidylinositol 3-kinase; IGF-1, insulin-like growth factor 1; GH, growth hormone; DTT, dithiothreitol; AT1 receptor, AII-receptor 1.

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rodent chow and water *ad libitum*, but food was withdrawn 12–14 h before the experiments. The rats were anaesthetized with sodium amobarbital (15 mg/kg body weight, intraperitoneally) and used in experiments 10–15 min later, as soon as anaesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the vena cava exposed and 0.5 ml of normal saline (0.9 % NaCl), with or without 10^{-5} M insulin or 10^{-8} M AII, was injected. After 1 min, the hearts were removed, minced coarsely and immediately homogenized in approx. 6 vol. of solubilization buffer A using a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 s in a water bath maintained at 100 °C as previously described [22,23]. The solubilization buffer A consisted of 1 % SDS, 50 mM Hepes (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA and 10 mM sodium vanadate. The homogenate was then boiled for 10 min and cooled in an ice bath for 40 min. In some experiments, the hearts were excised and homogenized with a Polytron in 6 vol. of homogenization buffer B cooled in an ice bath. The composition of buffer B was the same as buffer A except that 1 % Triton-X 100 replaced 1 % SDS and 2 mM PMSF and 0.1 mg/ml aprotinin were added. Both extracts were centrifuged at 100000 ***g*** (55000 rev./min) at 4 °C in a Beckman 70.1 Ti rotor for 60 min to remove insoluble material, and the resulting supernatant was used for the experiments. The hearts homogenized in buffer B were used for immunoprecipitation with anti-IRS-1 antibody and Protein A–Sepharose 6 MB.

Protein analysis by immunoblotting

The samples were treated with Laemmli sample buffer [24] containing 100 mM DTT and heated in a boiling-water bath for 4 min. For total extracts, similar size samples (150 μg of protein) were subjected to SDS/PAGE (6 or 7.5 % Tris/acrylamide) in a Bio-Rad miniature slab gel apparatus. Electrotransfer of proteins from the gel to nitrocellulose was performed for 2 h at 100 V (constant) in the Bio-Rad miniature transfer apparatus (Mini-Protean), as described by Towbin et al. [25] but with 0.02 % SDS added to the transfer buffer to enhance the elution of high-molecular-mass proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4 °C in blocking buffer (3 % BSA, 10 mM Tris, 150 mM NaCl and 0.02 % Tween 20). The prestained molecular-mass standards used were myosin (205 kDa), β-galactosidase (116 kDa), BSA (80 kDa) and ovalbumin (49.5 kDa).

The nitrocellulose blot was incubated with anti-phosphotyrosine antibodies or with anti-(PI 3-kinase) antibodies (1:500 diluted in blocking buffer) for 4 h at 22 °C and then washed for 60 min with the blocking buffer without BSA. The blots were subsequently incubated with 2 mCi of ^{125}I-Protein A (30 μCi/μg) in 10 ml of blocking buffer for 1 h at 22 °C and washed again as described above for 2 h. ^{125}I-Protein A bound to the anti-phosphotyrosine and anti-(PI 3-kinase) antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −70 °C for 12–48 h. Band intensities were quantified by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

Perfusion of isolated rat heart

Isolated, perfused rat heart has been used extensively in studies concerned with the regulation of muscle metabolism [26]. The classical Langendorff preparation was used in the experiments described herein [27]. The aorta was cannulated and the coronary vessels were perfused by introducing perfusate into the aorta. A Krebs–Henseleit bicarbonate buffer, pH 7.4, equilibrated with O_2/CO_2 (19:1) at 37 °C was used as a perfusion medium. Insulin or AII, at the same concentrations as described previously, were infused in the aorta and 1 min later the heart was extracted from the Langendorff perfusion apparatus and homogenized in buffer B. The extracts were centrifuged and used for immunoprecipitation with anti-IRS-1 antibody as previously described.

Protein determination

Protein determination was performed by the Bradford dye method [28] using the Bio-Rad reagent and BSA as the standard.

RESULTS AND DISCUSSION

Normal rats were injected via the vena cava with 10^{-8} M AII or 10^{-5} M insulin, followed 1 min later by extraction and homogenization of the heart in boiling buffer contaning SDS. The proteins were separated and subjected to immunoblot analysis with monoclonal anti-phosphotyrosine antibody. Figure 1(a)

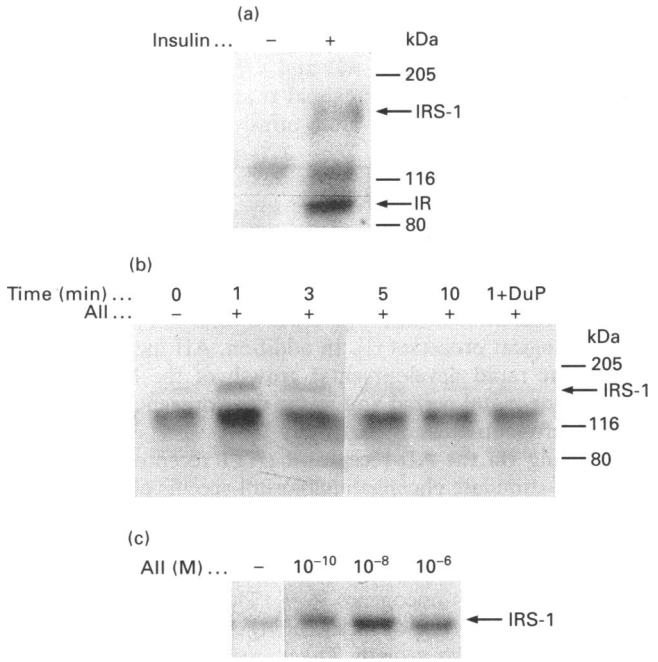

Figure 1 Insulin- and AII-stimulated tyrosine phosphorylation in intact heart of rat

(**a**) Rats were anaesthetized, and the abdominal wall incised to expose the viscera. Normal saline or 10^{-5} M insulin were infused into vena cava as a bolus injection and 1 min later hearts were excised and homogenized in extraction buffer A at 100 °C, as described in the Methods section. After centrifugation, aliquots with the same amount of protein were resolved on SDS/6 %-polyacrylamide gel, transferred to nitrocellulose and detected with anti-phosphotyrosine antibody and ^{125}I-Protein A and subjected to autoradiography. (**b**) The experiment was similar to (**a**) except that 10^{-8} M AII replaced 10^{-5} M insulin for the indicated times. 1 + DuP means that the animal was pretreated for 1 h with DuP 753 (10 mg/kg, subcutaneously), a non-peptide AT1 receptor antagonist, and then injected with 10^{-8} M AII, and 1 min later the heart was excised and homogenized as described. The proteins were resolved on SDS/7.5% PAGE, transferred to nitrocellulose and detected with anti-phosphotyrosine antibody and ^{125}I-Protein A and subjected to autoradiography. Data are representative of independent experiments that were performed at least three times. (**c**) Immunoblotting of tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates from heart of rats treated with three different doses of AII for 1 min. The heart proteins were extracted and processed as described in the Methods section, solubilized and incubated at 4 °C with anti-IRS antibody and Protein A–Sepharose 6MB. Immunoprecipitated proteins were analysed by immunoblotting with anti-phosphotyrosine antibody and ^{125}I-Protein A and subjected to autoradiography. Data are representative of three independent experiments.

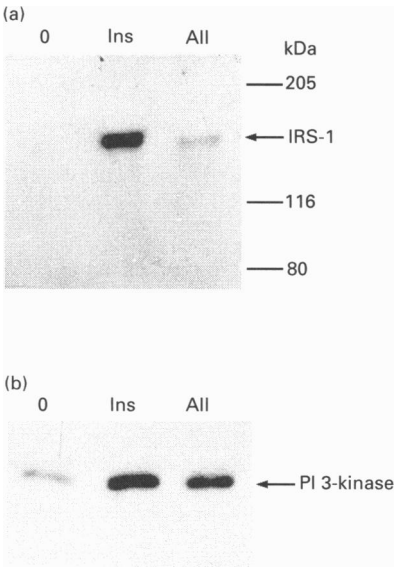

Figure 2 Immunoblotting of tyrosyl-phosphorylated proteins and PI 3-kinase (b) in anti-IRS-1 immunoprecipitates from heart of rats treated with saline (0), AII (10^{-8} M) or insulin (Ins) (10^{-5} M)

The heart proteins were extracted and processed as described in the Materials and methods section, solubilized and incubated at 4 °C with anti-IRS antibody and Protein A–Sepharose 6MB. Immunoprecipitated proteins were analysed by immunoblotting with anti-phosphotyrosine and anti-PI 3-kinase (85 kDa subunit) antibodies and ^{125}I-Protein A and subjected to autoradiography. Data are representative of independent experiments that were performed at least four times

shows that, after stimulation with insulin, a phosphotyrosine band of 95 kDa, previously identified as the insulin receptor β-subunit, appeared and became prominently phosphorylated. In addition, after injection of insulin, a phosphotyrosyl protein migrating between 165 and 185 kDa consistent with IRS-1, an endogenous substrate, was also detectable. AII stimulated the tyrosine phosphorylation of a prominent band migrating between 120 and 140 kDa and of a faint band of 170 kDa. Tyrosine phosphorylation of the 170 kDa band was maximal at 1 min and then reduced dramatically (Figure 1b). In some experiments a band of $\sim$ 80 kDa was also stimulated by AII.

To determine whether the 170 kDa band, which was tyrosine phosphorylated in response to AII, represented IRS-1, the animals were injected with insulin or AII and 1 min later the heart was excised and homogenized in Triton X-100 buffer containing phosphatase and protease inhibitors. Protein extracts were prepared under conditions that permitted immunoprecipitation with an anti-IRS-1 antibody (C-terminal) before immunoblot analysis using anti-phosphotyrosine antibody. Anti-IRS-1 antibody immunoprecipitated intense bands at 170 kDa from the hearts of animals which had been injected with insulin or AII (Figures 1c and 2a). Preliminary experiments using 10^{-10}–10^{-6} M AII indicated that the maximal response was obtained at 10^{-8} M (Figure 1c), and this concentration was used throughout all subsequent experiments. The phosphorylation of IRS-1 was much greater in response to insulin than to AII. Densitometry indicated a 5.6 ± 1.2-fold (mean $\pm$ S.D., $n = 5$) increase in the signal after AII and a 12.8 ± 3.1-fold ($n = 5$) increase after insulin (Figure 2a). These findings provide strong evidence that AII stimulates the phosphorylation of IRS-1. When the animals were pretreated for 1 h with DuP 753 (10 mg/kg, subcutaneously), a non-peptide AT1 receptor antagonist [29], there was a marked reduction in the AII-induced tyrosine phosphorylation of IRS-1 in whole-tissue extracts (Figure 1b) and also in samples previously immunoprectipitated with anti-IRS antibody (results not shown).

The AT1 receptor belongs to a family of receptors with seven transmembrane-spanning domains which are coupled to G-proteins [30]. The amino acid sequence of the AT1 receptor does not predict a tyrosine kinase protein, suggesting that the phosphorylation of IRS-1 induced by AII may depend on the activation of soluble membrane-associated tyrosine kinases, although the mechanism of coupling has not been elucidated. Using neonatal rat cardiac fibroblasts, it has recently been demonstrated that AII-induced tyrosine phosphorylation of some proteins is pertussis-toxin-sensitive, thus suggesting that this effect may be mediated by the G_i-dependent activation of a soluble tyrosine kinase [16].

In order to evaluate the specificity of the response to AII, we performed a similar experiment in rats, but instead of AII we injected adrenaline (epinephrine; 25 μg/100 g of rat) and extracted the hearts at times identical with those described in Figure 1(b). Adrenaline did not induce tyrosine phosphorylation of IRS-1, suggesting that the response observed after AII is not an effect of acute blood-pressure elevation on the myocardial IRS-1 response (results not shown).

IRS-1 has been shown to be phosphorylated in response to insulin [31–33], IGF-1 [18,34], interleukin 4 [35–37] and GH [20]. The present report is the first to demonstrate that AII stimulates the tyrosine phosphorylation of IRS-1. This finding suggests that IRS-1 phosphorylation may serve as a converging target in insulin-, IGF-1- and AII-stimulated signalling pathways and may ultimately result in cellular growth and metabolic activation. However, the function of IRS-1 in the AII-induced proliferation of cardiac fibroblasts and in overload hypertrophy is presently unknown.

Previous studies [23,38–43] have suggested that there is a relatively stable, high-affinity interaction between IRS-1 and the 85 kDa subunit of PI 3-kinase, such that both proteins are co-precipitated by antibodies to either protein. In heart samples previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antibodies directed against the 85 kDa subunit of PI 3-kinase, there was little or no detectable PI 3-kinase immunoreactivity in the basal state. Following stimulation with insulin or AII, a band with the expected molecular mass of the regulatory subunit of PI 3-kinase (85 kDa) was present in anti-IRS-1 antibody immunoprecipitates, a finding consistent with the formation of a stable association between IRS-1 and PI 3-kinase (Figure 2b). There was a 9.2 ± 2.1-fold ($n = 4$) increase in PI 3-kinase associated with IRS-1 after insulin stimulation and a 6.1 ± 1.2-fold ($n = 4$) increase after AII-stimulation.

Since AII may affect other mediators *in vivo* to induce these responses, we performed experiments where the effects of insulin or AII were analysed in perfused hearts. The results are presented in Figure 3. A clear stimulation in IRS-1 tyrosine phosphorylation and its association with PI 3-kinase was induced by insulin, and to a lesser extent by AII, showing that AII has a direct effect on this pathway. In accordance with this result is the finding that pretreatment of the animals with AT1 receptor antagonist markedly reduced the AII-induced tyrosine phosphorylation of IRS-1 (Figure 1b).

Interestingly, it has been demonstrated that AII can increase glucose transport and oxidation in skeletal muscle [44], thereby simulating insulin action. Moreover, it can improve insulin sensitivity in patients with non-insulin-dependent diabetes mellitus by an unknown mechanism that is not related to haemodynamic alterations and the redistribution of cardiac output [45]. There is increasing evidence that the IRS-1/PI 3-

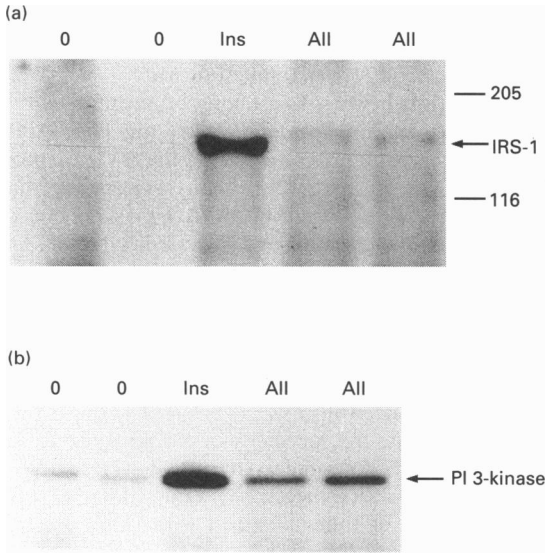

Figure 3 Immunoblotting of tyrosyl-phosphorylated proteins (a) and PI 3-kinase (b) in anti-IRS-1 immunoprecipitates from the isolated perfused heart of rats treated with saline (0), AII (10^{-8} M) or insulin (Ins) (10^{-5} M) into the perfusion buffer

The heart proteins were extracted and processed as described in the Materials and methods section, solubilized and incubated at 4 °C with anti-IRS antibody and Protein A–Sepharose 6MB. Immunoprecipitated proteins were analysed by immunoblotting with anti-phosphotyrosine (upper part of the filter) and anti-(PI 3-kinase) (lower part of the filter) antibodies and ^{125}I-Protein A and subjected to autoradiography. Data are representative of independent experiments that were performed at least twice.

kinase pathway may be linked to the activation of glucose transport [46–48]. On the basis of these results, it is tempting to speculate that IRS-1 phosphorylation and its association with PI 3-kinase may have a role in the stimulation of glucose transport induced by AII.

In summary, we have characterized an additional signalling mechanism stimulated by AII in the rat heart *in vivo*. Future studies, aimed at identifying activated intracellular protein tyrosine kinase(s) and their role in AII-initiated signal transduction, will provide a more complete understanding of the integrated cellular functions of AII in cardiovascular diseases.

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