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Cross-talk between the insulin and angiotensin signaling systems

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ABSTRACT **Angiotensin II (AII), acting via its G-protein linked receptor, is an important regulator of cardiac, vascular, and renal function. Following injection of AII into rats, we find that there is also a rapid tyrosine phosphorylation of the major insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) in the heart. This phenomenon appears to involve JAK2 tyrosine kinase, which associates with the AT1 receptor and IRS-1/IRS-2 after AII stimulation. AII-induced phosphorylation leads to binding of phosphatidylinositol 3-kinase (PI 3-kinase) to IRS-1 and IRS-2; however, in contrast to other ligands, AII injection results in an acute inhibition of both basal and insulin-stimulated PI 3-kinase activity. The latter occurs without any reduction in insulin receptor or IRS phosphorylation or in the interaction of the p85 and p110 subunits of PI 3-kinase with each other or with IRS-1/IRS-2. These effects of AII are inhibited by AT1 receptor antagonists. Thus, there is direct cross-talk between insulin and AII signaling pathways at the level of both tyrosine phosphorylation and PI 3-kinase activation. These interactions may play an important role in the association of insulin resistance, hypertension, and cardiovascular disease.**

Insulin resistance occurs in a wide variety of pathological states and is a central component of non-insulin dependent diabetes mellitus (1). The frequent clustering of insulin resistance, hypertension, central obesity, hypertriglyceridemia, and accelerated atherosclerosis has lead to the definition of a common metabolic condition often referred to as syndrome X (2, 3). Over the past decade, many of the proteins involved in insulin action have been defined at a molecular level (4). The insulin receptor is a protein tyrosine kinase which, when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including Shc, one or more 50–60 kDa proteins, and two related high molecular weight insulin receptor substrates, IRS-1 and IRS-2 (4, 5). Following tyrosine phosphorylation, IRS-1 and IRS-2 act as docking proteins for several Src homology 2 domain-containing molecules, including phosphatidylinositol 3-kinase (PI 3-kinase), Grb2, SHPTP2, NCK, and Fyn (4, 6, 7). The interaction between the IRS proteins and PI 3-kinase occurs through the p85 regulatory subunit of the enzyme and results in an increase in catalytic activity of the p110 subunit (6, 8). PI 3-kinase is essential for many insulin-sensitive metabolic processes including stimulation of glucose transport, activation of the p70 S6 and Akt serine kinases, and stimulation of glycogen and protein synthesis (9–13).

Angiotensin II (AII) plays an important role in cardiovascular and neuroendocrine physiology and fluid volume homeostasis, and may also act as a growth factor for heart and vascular smooth muscle (14). Angiotensin-converting enzyme inhibitors are a cornerstone in the therapy of human hypertension and cardiac failure (15). Most of the known actions of AII are exerted through the AT1 receptor, a G-protein-coupled receptor, which is linked to the stimulation of phospholipase C (16). In addition, AII stimulation results in activation of mitogen-activated protein kinase (17), and, as we and others have recently shown, stimulation of tyrosine phosphorylation of several proteins such as IRS-1 (18), Shc (14), paxillin (19), and the STAT transcription factors (20), which are normally involved in the action of various growth factors, including insulin. In view of the important association between insulin resistance, hypertension, and cardiovascular disease, in this study we have explored the interaction between the insulin and angiotensin signaling systems in the intact rat and found both positive and negative cross-talk between these two hormonal response pathways.

MATERIALS AND METHODS

Antibodies and Chemicals. Antibodies against IRS-1, IRS-2, and the insulin receptor were prepared as described (5, 21). Polyclonal antibodies against JAK2, AT1 receptor, and the p110α catalytic subunit of PI 3-kinase were from Santa Cruz Biotechnology. Monoclonal antibodies against phosphotyrosine (4G10) and polyclonal antibodies against the p85 regulatory subunit of PI 3-kinase were from Upstate Biotechnology. Affinity-purified rabbit anti-mouse IgG was from Dako. [^{125}I]protein-A and [^{32}P]ATP were from Amersham and NEN/DuPont, respectively. Protein A-Sepharose 6MB was from Pharmacia. Rat liver phosphatidylinositol was from Avanti Polar Lipids. All other reagent grade chemicals were from Sigma.

Animals. Male Wistar rats (180–200 g) were allowed free access to standard rodent chow and water ad libitum. Food was withdrawn 12 hr before the experiments. Rats were anesthetized by intraperitoneal injection of sodium amobarbital (100 mg/kg body wt), and the experiments were performed after the loss of corneal and pedal reflexes.

AII and Insulin Treatment and Tissue Preparation. *In vivo* stimulation of the heart was obtained by injection of 200-μl solutions containing either AII, insulin, or saline into the inferior vena cava. The tip of the left ventricle was excised after 60 sec and immediately homogenized (Polytron PTA 20S generator, Brinkmann) in freshly prepared 50 mM Hepes buffer (pH 7.4) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM EDTA, 2 mM phenylmethylsulfonyl flouride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM benzamidine, 10% (vol/vol) glycerol, and 1% (vol/vol) Nonidet P-40; insoluble material was removed by centrifugation for 45 min at 15,000 rpm in a 70.Ti rotor (Beckman) at 4°C. The protein concentration of the supernatants was determined by the Bradford method (45).

PI 3-Kinase Assay. Aliquots of supernatants containing equal amounts of proteins were incubated overnight at 4°C

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Abbreviations: AII, angiotensin II; IR, insulin receptor; IRS-1 and IRS-2, insulin receptor substrates 1 and 2; PI 3-kinase, phosphatidylinositol 3-kinase.

†L.A.V. and F.F. contributed equally to this paper.

¶C.R.K. and M.J.A.S. were co-advisors in these studies.

using antibodies against IRS-1, IRS-2, or the p85 subunit of PI 3-kinase, and the immunocomplexes were precipitated with a 50% solution of protein A-Sepharose 6MB. *In vitro* PI 3-kinase assays were performed as described (6). The ^{32}P-labeled 3-P-phosphatidylinositol was quantitated by the IMAGEQUANT software of the PhosphorImager (Molecular Dynamics).

Immunoprecipitations and Western Blotting. Tyrosine phosphoproteins and other specific proteins were detected by immunoprecipitation followed by SDS/PAGE and Western blotting as described (6, 21). An amplification step with rabbit anti-mouse IgG (1:1000 final dilution) was added to the standard protocol when 4G10 monoclonal anti-phosphotyrosine antibodies were employed in Western blotting experiments.

Statistics. For comparison of data presented as mean ± SEM, the paired Student's *t* test was used.

RESULTS

Effects of AII and Insulin on Tyrosine Phosphorylation, JAK Kinase, and PI 3-Kinase Docking. To explore the interactions between the insulin and AII signaling pathways *in vivo*, rats were injected with insulin and/or AII and the early steps in signal transduction were assessed using extracts of heart, a tissue which responds to both hormones. Immunoprecipitation of extracts with anti-IRS-1 or anti-IRS-2 antisera, followed by immunoblotting with anti-phosphotyrosine antibodies, revealed a rapid phosphorylation of IRS-1 and IRS-2 following injection of either insulin or AII (Fig. 1 *A* and *B*). The increase in phosphorylation was accompanied by an association of both IRS-1 and IRS-2 with the enzyme PI 3-kinase as determined by immunoblotting of the complex with antibodies to either the regulatory (p85) or catalytic (p110) subunits of the protein (Fig. 1 *C–F*). Quantitation of multiple experiments revealed that AII increased the tyrosyl phosphorylation of IRS-1 and IRS-2 by 2.6- and 3.8-fold, respectively, and increased the association of these proteins with the p85 regulatory and p110α catalytic subunits of PI 3-kinase by about 3- to 4-fold. Under the same conditions, insulin induced a 6- to 8-fold increase in the tyrosine phosphorylation of IRS-1 and IRS-2 and a parallel increase in their association with PI 3-kinase.

In addition to insulin and IGF-1, which act via tyrosine kinase receptors, several cytokines, interferons, and growth hormones also stimulate phosphorylation of IRS-1 and IRS-2 through receptors that lack intrinsic kinase activity, but appear to recruit cytoplasmic tyrosine kinases of the Janus kinase (JAK) family (5, 22, 23). Recent studies suggest that AII can also stimulate JAK2 kinase (20). To determine the role of JAK kinases in tyrosine phosphorylation of IRS-1/IRS-2, we performed immunoprecipitation of cardiac muscle extracts using anti-JAK2 antibody and immunoblotting with anti-phosphotyrosine antibody. After AII or insulin stimulation, the anti-JAK2 immunoprecipitates contained phosphotyrosyl proteins of ≈180 and 130 kDa not observed in the saline-treated controls (Fig. 2*A*). Reprobing of the filters with specific antibodies indicated that the ≈180-kDa protein corresponds to IRS-1 and

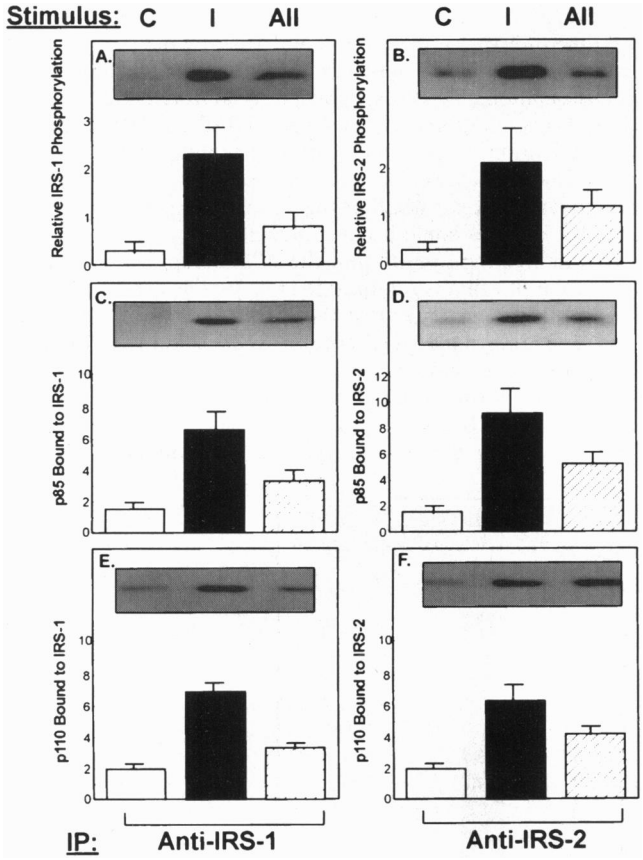

FIG. 1. IRS-1 or IRS-2 phosphorylation and PI 3-kinase binding in rat heart after saline (C), insulin (I), or AII (AII) treatment. Rats were injected with saline or 200 μl of 10^{-6} M insulin or 10^{-8} M angiotensin. Tissues were extracted and immunoblots prepared as described. (*A* and *B*) Nitrocellulose transfers blotted with anti-phosphotyrosine antibody and the tyrosine phosphorylated band corresponding to IRS-1 (*A*) and IRS-2 (*B*). The nitrocellulose transfers were stripped and reblotted with antibodies to the p85 (*C* and *D*) and p110α subunits (*E* and *F*) of PI 3-kinase. The bar graphs show the relative phosphorylation of IRS-1 and IRS-2 and the binding of p85 and p110α as determined by PhosphorImager quantitation. Data are presented as mean ± SEM. Stimulation was significant ($P < 0.05$) as determined using a paired Student's *t* test ($n = 5$).

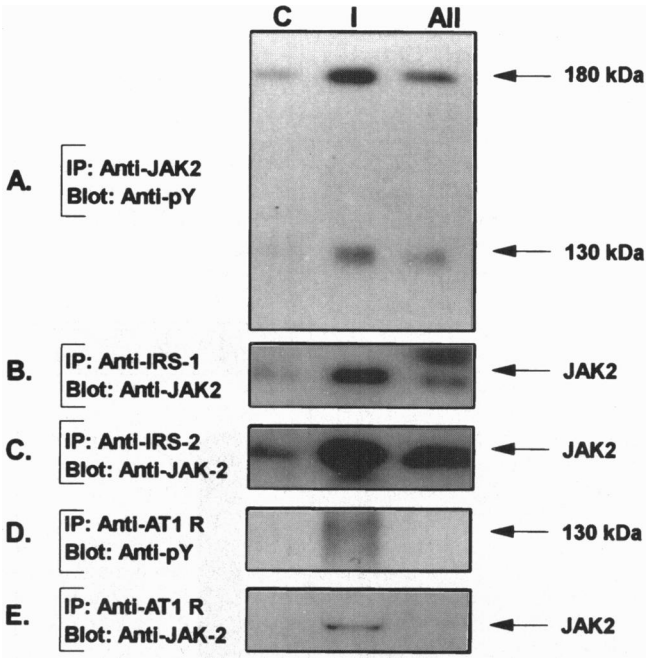

FIG. 2. Autoradiograms of SDS/PAGE of JAK2 immunoprecipitates from rat heart after saline (C), 10^{-5} M insulin (I), or 10^{-8} AII (AII) treatment. The animals were treated as described in Fig. 1, except that the extracts were precipitated with anti-JAK2 antibody. The nitrocellulose filters were then blotted with anti-phosphotyrosine antibody, followed by rabbit anti-mouse IgG (1:1000, final dilution) and ^{125}I-protein-A. The bands appearing at 180 and 130 kDa were confirmed to be IRS-1/IRS-2 and JAK2 by stripping the filter and reblotting with anti-IRS-1, anti-IRS-2, and anti-JAK2 antibodies. In parallel complementary experiments, the extracts were immunoprecipitated with anti-IRS-1 (*B*) or anti-IRS-2 (*C*), run out on SDS gels, transferred to nitrocellulose, and blotted with the anti-JAK2 antibody. (*D* and *E*) Immunoprecipitation with anti-AT1 receptor antibody followed by blotting with anti-phosphotyrosine and anti-JAK2 antibodies.

IRS-2, whereas the 130-kDa protein corresponds to JAK2 (not shown). Direct immunoprecipitation with anti-IRS-1 or anti-IRS-2 antisera followed by blotting with anti-JAK2 antiserum confirmed the association of IRS-1 and IRS-2 with JAK2 following AII or insulin treatment (Fig. 2 *B* and *C*). Likewise, immunoprecipitation of rat heart extracts using anti-AT1 receptor antibody followed by blotting with anti-JAK2 antibody revealed that JAK2 co-precipitated with the AT1 receptor, and that tyrosine phosphorylation of JAK2 in the AT1 receptor immunoprecipitates was stimulated by AII (Fig. 2 *D* and *E*).

Inhibition of PI 3-Kinase by AII. Numerous previous studies using insulin, IGF-1, and other ligands have shown that following binding of p85 to phosphorylated IRS-1 or IRS-2, the catalytic activity of PI 3-kinase is stimulated several-fold (5, 6, 8). Similarly, following insulin injection into rats, there were 4.2-fold ($P < 0.05$) and 8-fold ($P < 0.005$) increases in IRS-1- and IRS-2-associated PI 3-kinase activity in cardiac muscle (Fig. 3). Surprisingly, however, following AII injection there was a dose-dependent inhibition of PI 3-kinase activity associated with IRS-1 and IRS-2, with maximal decreases of 55% ($P < 0.05$) and 60% ($P < 0.01$) below basal values after injection of as little as 200 pmol of AII (Fig. 3).

The ability of AII to inhibit PI 3-kinase activity was also observed during insulin stimulation (Fig. 4). When AII (10^{-8} M) was infused 1 min before insulin, the levels of IRS-1- and IRS-2-associated PI 3-kinase activity were reduced by 74% ($P < 0.01$) and 86% ($P < 0.005$), respectively, as compared with insulin treatment alone. Inhibition was also observed with simultaneous administration of both insulin and AII, although the effect was slightly smaller (data not shown). Infusion of a peptide blocker of the AT1 receptor 1 min before AII treatment completely prevented AII-induced IRS-1 phosphorylation (data not shown), as well as the inhibitory effect of AII on insulin stimulated, IRS-1-associated PI 3-kinase activity, but had no effect on basal or insulin-stimulated PI 3-kinase activity (Fig. 4 *B* and *C*). This is in agreement with our previous observation that DUP 753 (Losartan), a non-peptidal blocker of the AT1 receptor, also leads to a reduction in the AII-induced tyrosine phosphorylation of the IRS-1 (18). The ability of AII to inhibit insulin-stimulated PI 3-kinase activity occurred without any effect of AII to alter insulin stimulation of insulin receptor or IRS-1/IRS-2 phosphorylation and with no significant reduction in the binding of p85 to IRS-1/IRS-2 or p110 to p85 (Fig. 5).

DISCUSSION

Intracellular interactions between different signaling systems may function as mechanisms of enhancement or counter-regulation of hormone action. Recent evidence indicates that ligands signaling through G-protein-coupled receptors may mimic some effects classically observed after activation of tyrosine kinase receptors, including activation of mitogen-activated protein kinase, PI 3-kinase, pp125^{FAK}, JAK2, and phosphotyrosine phosphatases (17, 20, 24). This study demonstrates that in the case of AII, the cross-talk with typical tyrosine kinase-mediated pathways are multiple and result in direct interactions between the AII and insulin signaling systems. AII is able to induce the rapid tyrosine phosphorylation of IRS-1 and IRS-2 in rat heart in a physiologic dose-responsive manner. This phosphorylation probably occurs in the cardiac myocytes themselves, because immunocytochemical experiments reveal that these cells are major sites of expression for IRS-1 (F.F. and C.R.K., unpublished observation) and angiotensin receptors (25) in heart. This phosphorylation event appears to be mediated by JAK2, which undergoes rapid phosphorylation following AII stimulation and associates with the AII receptor and IRS-1/IRS-2 (ref. 20 and this study). Insulin (Fig. 2 *B* and *C*), growth hormone (26), and interleukins (23) also stimulate JAK2 phosphorylation and association with tyrosyl phosphorylated IRS-1 and IRS-2. Thus, the association between JAK2 and IRS-1/IRS-2 appears to be common to phosphorylation stimulated by several tyrosine kinases, as well as non-tyrosine kinase receptors, although it is not clear whether this interaction is direct or if this complex involves some other molecule(s).

It is interesting to note that the AT1 receptor possesses an NPXXY motif that is required for many of its actions (16, 27). This motif is reminiscent of the NPXY motif present in the insulin and interleukin 4 receptors, which is required for IRS-1 phosphorylation and downstream signaling and, in the insulin receptor, is phosphorylated in response to hormonal stimulation (28, 29). Following AII stimulation, the AT1 receptor undergoes both serine and tyrosine phosphorylation (30),

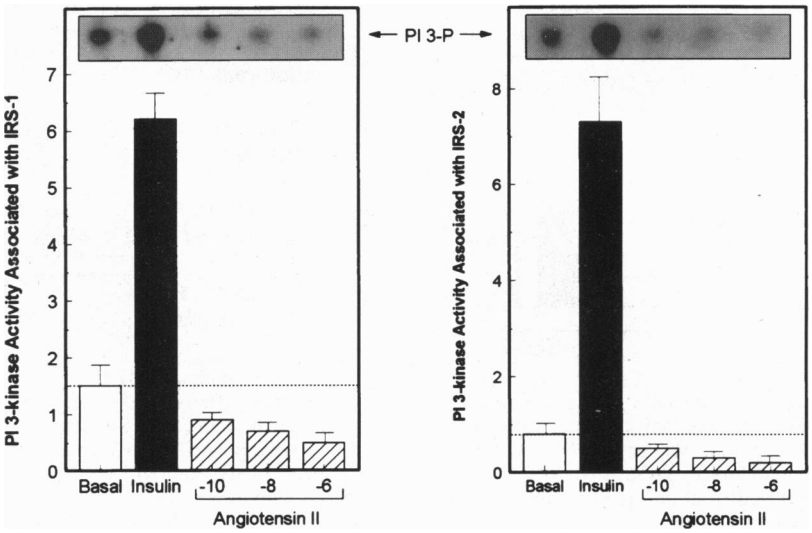

FIG. 3. IRS-1- and IRS-2-associated PI 3-kinase activity in rat heart after saline (C), insulin (I), and AII treatment. Rats were injected with hormones as described, and the hearts extracted and immunoprecipitated overnight using antibodies against IRS-1 or IRS-2. PI 3-kinase assays were performed as described (6). Fluorographs show the silica TLC plates of IRS-1- (*Left*) and IRS-2- (*Right*) associated PI 3-kinase activity. PI 3-P indicates the migration position of phosphatidylinositol 3-phosphate. Bar graphs depict the relative incorporation of ^{32}P into PI 3-P (mean ± SEM) from five separate experiments. The inhibition by 10^{-10} and 10^{-8} M AII was significant at $P \leq 0.05$, in comparison with saline-injected controls. The inhibition at 10^{-6} M AII was significant at $P < 0.01$.

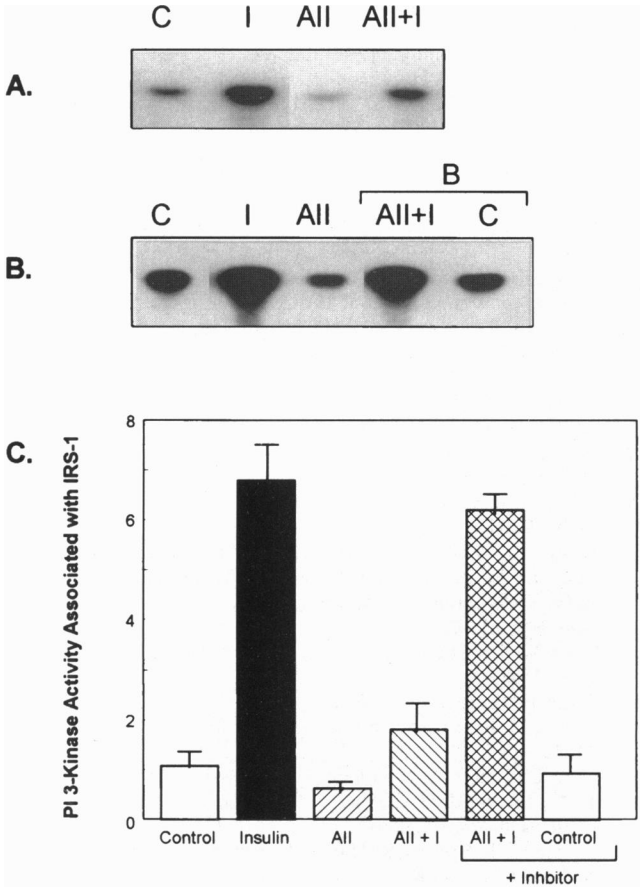

FIG. 4. Effect of insulin and AII on PI 3-kinase activity and the role of AT1 receptor. Animals were injected with AII or insulin alone, or sequentially with AII and then insulin 1 min later. Tissues were extracted and assayed as described. (*A* and *B*) Fluorographs of silica TLC plates of IRS-1-associated PI 3-kinase activity in rat heart after saline (C), 10^{-5} M insulin (I), 10^{-8} M AII (AII), or 10^{-8} M or AT1 receptor peptide inhibitor (QGVYVHPV, Sigma) treatment. (AII + I) Animals that received 10^{-8} M AII 1 min before the injection of 10^{-5} M insulin. The bar graph in *C* depicts the relative incorporation of ^{32}P in PI 3-P (mean ± SEM) of four separate experiments, the reversal of the AII effect by the receptor. Inhibitor was significant at the $P < 0.05$ level.

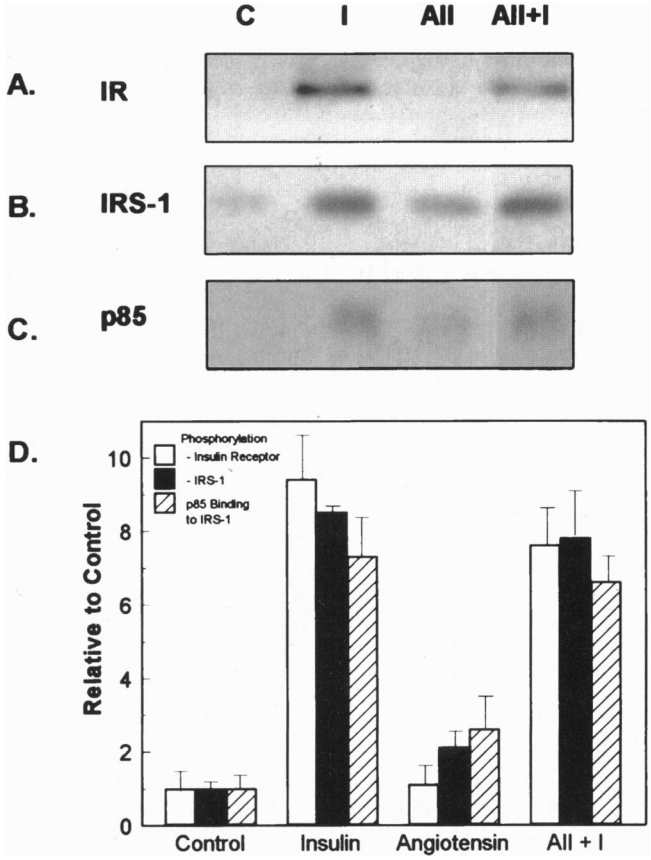

FIG. 5. Lack of effect of AII on insulin-stimulated receptor and IRS-1 phosphorylation and p85 binding. (*A* and *B*) Anti-phosphotyrosine immunoblots of anti-insulin receptor (IR) and anti-IRS-1 immunoprecipitates from rat hearts after saline (C), 10^{-5} M insulin (I), 10^{-8} AII (AII), or treatment with a sequential infusion of AII and insulin with a 60-sec interval between each infusion. (*A* and *B*) Bands appearing at 95 kDa and 165–185 kDa are shown as IR and IRS-1, respectively. (*C*) Lower part of the nitrocellulose transfer from the anti-IRS-1 preciptitates was blotted with anti-p85 antibody. (*D*) Data of six replicate experiments has been quantitated by PhosphorImager analysis and are shown as mean + SEM. None of the differences in insulin-receptor phosphorylation, IRS-1 phosphorylation, or p85 binding to IRS-1, are statistically significant between the insulin alone group and the insulin plus AII group.

although no data are available at present as to whether this occurs at the NPXXY site. Nonetheless, it is tempting to hypothesize that phosphorylation of the AT1 receptor at the NPXXY motif might be necessary for JAK2/IRS1-2/PI 3-kinase mediated signaling.

In all cases in which there is stimulation of tyrosine phosphorylation of IRS-1 and IRS-2, there is a concomitant docking of these proteins to the p85 subunit of PI 3-kinase. In all previous cases, this docking was associated with a stimulation of the PI 3-kinase enzyme activity. What was surprising in this study, however, is that IRS-1- and IRS-2-associated PI 3-kinase activity is inhibited following AII injection rather than stimulated. This effect is direct in that it occurs acutely, and both IRS-1 and IRS-2 phosphorylation and the inhibition of PI 3-kinase activity can be blocked by inhibitors of the AT1 receptor (see ref. 18 and this study). This inhibitory phenomenon is also observed in liver (data not shown).

Interactions between G-protein-coupled receptors and tyrosine kinase signaling pathways have been previously reported. Tyrosyl phosphorylation of the β2-adrenergic receptor by insulin has been shown to produce supersensitization of adrenergic signaling (31), whereas protein kinase A activation leads to attenuation of insulin- and epidermal growth factor-stimulated mitogen-activated protein kinase activity (32). Insulin receptors are capable of tyrosine phosphorylating G_i, G_0, and transducin *in vitro* (33, 34), and we and others have previously shown that insulin is able to inhibit ADP ribosylation of G_i (35, 36). $G_{i\alpha 2}$ deficiency in liver and adipose tissue of transgenic mice has also been shown to result in impaired glucose tolerance and insulin resistance associated with attenuated insulin-stimulated tyrosine phosphorylation of IRS-1 (37).

Our studies show a new and more direct level of interaction between receptor tyrosine kinases and receptors acting through G-proteins with phosphorylation of a substrate of the insulin receptor following AII stimulation (see ref. 18 and this study). More importantly, in this study we find the first evidence of a reduction of PI 3-kinase activity after its association with a tyrosyl-phosphorylated docking protein. The precise mechanism of this inhibition is unknown. It is not due to a dissociation of the p85 and p110 subunits of PI 3-kinase. Other possibilities would include differential sites of tyrosyl phosphorylation on IRS-1 and IRS-2 proteins following AII and insulin treatment, recruitment of an inhibitor of PI-3 kinase activity to the IRS-1 complex by AII, or some covalent modification of p85 or p110 that alters enzymatic activity. Of these possibilities, the latter seems most likely since it has been

shown *in vitro* that phosphorylation of p85 on Ser-608 reduces the catalytic activity of PI 3-kinase (38). Furthermore, using rat aortic smooth muscle cells in culture, we find that AII inhibition of PI 3-kinase activity is associated with a 50% increase in the serine phosphorylation of the p85 regulatory subunit. This phenomenon is also observed following phorbol 12-myristate 13-acetate stimulation suggesting a possible role for protein kinase C in the inhibitory effect of AII- on IRS-associated PI 3-kinase activity (F.F., C.R.K., and E. P. Feener, unpublished work). In adipocytes, insulin-stimulated PI 3-kinase activation is crucial for glucose transport, cell growth, protein synthesis, and stimulation of the 70-kDa S6 kinase (9, 10, 13). In heart, despite the inhibition of PI 3-kinase activity, AII stimulates cellular hypertrophy and growth acting through both the mitogen-activated protein kinase and 70-kDa S6 kinase pathways (39). Thus, cross-talk between AII and insulin signaling must take place at several levels.

The implications of intracellular interactions between the AII and insulin signaling systems are many. It seems likely that this interaction is physiologically important, because it occurs at physiological concentrations of hormone in intact animals and because one of the most common disease associations in humans is between diabetes, insulin resistance, and hypertension. Furthermore, both insulin and angiotensin have effects on cardiac and vascular smooth muscle growth and hypertrophy, and the insulin resistance/hypertension syndrome is associated with accelerated atherosclerosis (2, 3, 14). Administration of angiotensin-converting enzyme inhibitors and AT1 receptor blockers to both normal and hypertensive individuals has also been shown to result in improved insulin sensitivity and even hypoglycemia, strongly suggesting an association between elements involved in glucose homeostasis control and blood pressure (40–42). Recent evidence also suggests that insulin-mediated vasodilation contributes to increased insulin sensitivity and responsiveness in human skeletal muscle (43). Thus, part of the insulin "sensitizing" effect of angiotensin-converting enzyme inhibitors might be ascribed to vasodilation, especially in the face of the increased vascular tone, typical of hypertension. Furthermore, in hyperinsulinemic clamp studies, although AII infusion results in decreased glucose extraction across the leg, this is more than compensated for by the increase in blood flow to the same tissue (44).

In summary, intracellular cross-talk between AII and insulin signaling may be of pathophysiologic significance in the insulin resistance associated with hypertension and accelerated atherosclerosis. The potential for this interaction as a site for new therapeutic approaches of insulin resistance, as well as for increased understanding of the insulin signaling and G-protein mediated pathways, deserves further exploration.

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