Insulin Induces Tyrosine Phosphorylation of JAK2 in Insulin-sensitive Tissues of the Intact Rat*

(Received for publication, April 17, 1996, and in revised form, May 31, 1996)

Mario J. A. Saad†, Carla R. O. Carvalho, Ana C. P. Thirone, and Lício A. Velloso

From the Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, UNICAMP, Campinas, SP, Brazil 13081-970

The Janus kinase family of protein tyrosine kinases constitutes a novel type of signal transduction pathway activated in response to a wide variety of polypeptide ligands and has four known members: JAK1, JAK2, JAK3, and Tyk2. In this study, we examined the ability of insulin to stimulate JAK2 tyrosine phosphorylation in insulin-sensitive tissues of the intact rat using immunoprecipitation and immunoblotting. The results demonstrate that after an infusion of insulin, JAK2 is rapidly tyrosine phosphorylated (and the kinase is activated) in the liver, adipose tissue, skeletal muscle, heart, and isolated adipocytes. The presence of phosphorylated JAK2 was detectable after an infusion of insulin that increased serum insulin to physiological postprandial levels (40–70 microunits/ml). Co-immunoprecipitation with anti-insulin receptor antibody, anti-JAK2 antibody, and anti-IRS-1 antibody showed that JAK2 interacts with the insulin receptor and IRS-1 to form stable complexes following stimulation by insulin. In two animal models of insulin resistance the regulation of JAK2 tyrosine phosphorylation after insulin infusion paralleled the phosphorylation of the insulin receptor and of IRS-1. In conclusion, our data indicate that after physiological stimulation by insulin in the intact animal, JAK2 associates with the insulin receptor and is tyrosine phosphorylated in insulin-sensitive tissues in a time- and dose-dependent fashion.

The insulin receptor is the principal mediator of insulin action on cellular mitogenic and metabolic processes. The insulin receptor β-subunit, which contains an intrinsic tyrosine kinase, undergoes tyrosyl autophosphorylation and is activated in response to insulin binding to the extracellular α-subunit. Moreover the discovery of the insulin receptor's tyrosine kinase activity suggested that the mechanism of insulin action involved the tyrosyl phosphorylation of intracellular substrates. Using anti-phosphotyrosine antibodies an insulin-stimulated phosphoprotein called pp185 was identified in many cells and tissues (1, 2). One component of the pp185 band was purified and cloned from several sources (3–8). The cloned protein was called insulin receptor substrate 1 (IRS-1). More recently another constituent of the pp185 band termed IRS-2 was also purified, and its cDNA sequence was determined (9). There is also evidence showing that the protein Shc is tyrosine phosphorylated in response to insulin (10–13). Other proteins such as ecto-ATPase (14) and pp60 (15) are also known to be phosphorylated following insulin treatment. Furthermore a direct interaction between insulin receptor and phosphatidylinositol 3-kinase (16, 17) in addition to the interaction/activation of the latter with insulin receptor substrates 1 and 2 (9, 18) has been demonstrated.

The Janus kinase (JAK) family of protein tyrosine kinases constitute a novel type of signal transduction pathway activated in response to a wide variety of polypeptides ligands. The JAK family of nonreceptor protein tyrosine kinases has four known members: JAK1 (19), JAK2 (20, 21), JAK3 (22, 23), and Tyk2 (24). Each protein is ~130 kDa in mass and has a C-terminal tyrosine kinase domain, an adjacent kinase-related domain, and five further domains with amino acid similarity between members of the family extending toward the N terminus (20). Different cytokines and polypeptides hormones activate different JAKs. The receptors for erythropoietin (25), prolactin (26, 27), growth hormone (28), and angiotensin II (29) each have been demonstrated to bind and activate JAK2. However, the effect of insulin on JAK2 tyrosine phosphorylation and its association with the insulin receptor have not yet been investigated. In this report, we have examined the ability of insulin to stimulate JAK2 phosphorylation in insulin-sensitive tissues of the intact rat after injection of the hormone. Our data indicate that following stimulation by insulin JAK2 associates with the insulin receptor and is tyrosine phosphorylated in the liver, heart, adipose tissue, and skeletal muscle in a time- and dose-dependent fashion.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad. Aprotinin, ATP, dithiothreitol, HEPES, phenylmethylsulfonyl fluoride, angiotensin II, Triton X-100, Tween 20, glycerol, and bovine serum albumin (BSA, fraction V) were from Sigma. Protein A-Sepharose 6MB was from Pharmacia Biotech Inc., 125I-protein A was from ICN Biomedicals (Costa Mesa, CA), nitrocellulose paper (BA85, 0.2 mm) was from Schleicher & Schuell, and [γ-32P]ATP was from Amersham Corp. 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 (YIPGATMGTSTALTGDEAA) derived from the last 15 amino acids of the C terminus of rat IRS-1 as described previously (30). Anti-phosphotyrosine monoclonal and anti-JAK2 polyclonal antibodies were from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA). Anti-insulin receptor antibody was raised in rabbits using a synthetic peptide derived from the amino acid sequence (KKNGRILTLPRSNPS) corresponding to the C terminus of the protein.

**Methods**

Animals and Tissue Extracts—Male rats (130–180 g) were allowed access to standard rodent chow and water ad libitum. Food was withdrawn 12–14 h before the experiments. The rats were anesthetized with sodium amobarbital (15 mg/kg body weight, intraperitoneally) and used 10–15 min later, as soon as anesthesia was assured by the loss of pedal...
and corneal reflexes. The abdominal cavity was opened, the portal vein (liver) or the vena cava (adipose tissue, heart, and skeletal muscle) as a bolus injection. One minute later the tissues were excised and homogenized in extraction buffer at 4 °C as described under “Experimental Procedures.” After centrifugation, aliquots containing equal amounts of protein were immunoprecipitated with anti-JAK2 antibody and protein A-Sepharose 6MB and then resolved on 6% SDS-polyacrylamide gels. The protein bands were subsequently transferred to a nitrocellulose membrane and detected with anti-phosphotyrosine antibody and 125I-protein A, after which the membrane was subjected to autoradiography. The data are representative of three experiments.

Cell Incubations—Epididymal and perirenal fat were harvested from 130–180-g rats. Adipocytes were isolated by collagenase digestion as described previously (31, 32) and after being washed free of enzymes other tissue such as adipose tissue and skeletal heart muscle were also extracted. No more than two tissues were processed from the same animal. For the time course and dose-response experiments with adipose tissue, skeletal muscle, and heart tissue, insulin was injected into the vena cava. The extracts were centrifuged at 55,000 rpm at 4 °C in a Beckman 70.1 Ti rotor for 60 min to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with anti-JAK2, anti-insulin receptor, and anti-IRS-1 antibodies. The immune complexes were precipitated with protein A-Sepharose 6MB and were washed three times with 50 mM Tris (pH 7.4), 10 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, and 0.1% Triton X-100. Aliquots of the cell suspension (600 μl) were incubated with insulin (400 ng/ml) or saline in 1.5-ml plastic centrifuge tubes for 1 min. The incubation was terminated by centrifuging for 5 s and aspirating the incubation medium. Proteins were extracted by adding 600 μl of the extraction buffer as described above. The extracts were centrifuged and used for immunoprecipitation with anti-JAK2 antibody.

Protein Analysis by Immunoblotting—After washing, the pellet was resuspended in Laemmli sample buffer (33) with 100 mM dithiothreitol and heated in a boiling water bath for 4 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (6% Tris acrylamide) in a Bio-Rad miniature slab gel apparatus. Electrophoresis of proteins from the gel to nitrocellulose was performed for 2 h at 100 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protein) as described by Towbin et al. (34) but with 0.02% SDS added to the transfer buffer to enhance the elution of high molecular mass proteins. Nonspecific protein binding to the nitrocellulose was reduced by preincubating 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 (194 kDa), β-galactosidase (116 kDa), bovine serum albumin (85 kDa), and ovalbumin (49.5 kDa).

The nitrocellulose blot was incubated with anti-phosphotyrosine antibodies or with the appropriate antibody diluted in blocking buffer for 4 h at 22 °C and washed for 60 min with the blocking buffer without BSA. The blots were then incubated with 2 μCi of 32P-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. 32P-Protein A bound to the anti-phosphotyrosine or other 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 quantitated by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

JAK2 in Vitro Kinase Assay—Immunoprecipitated proteins bound to protein A-Sepharose were washed with kinase buffer (50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 0.1 mM Na3VO4, and 10 mM HEPES, pH 7.4) and subsequently incubated for 30 min at 24 °C with kinase buffer containing 0.25 mCi/ml [γ-32P]ATP. After thorough washing with kinase buffer, the proteins were eluted by boiling with Laemmli sample buffer and separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described above (19, 21).

JAK2 tyrosine kinase activity was also measured by autophosphorylation using a different approach as described previously (29). Insulin was infused into the portal vein to stimulate limited receptor activation and partial JAK2 autophosphorylation. JAK2 was then immunoprecipitated and allowed to autophosphorylate in vitro in the presence of exogenous ATP. Tyrosine phosphorylation was measured by immunoblotting with anti-phosphotyrosine antibody.

Other—Protein determination was performed by the Bradford dye method (35) using the Bio-Rad reagent and BSA as the standard.

Statistical Analysis—Experiments to investigate the regulation of

**Fig. 1.** Insulin-stimulated JAK2 tyrosine phosphorylation in rat tissues. Rats were anesthetized, and the abdominal wall was incised to expose the viscera. Saline, 2 μg of insulin, or 10 mM angiotensin II (as depicted in the figure) were administered into the portal vein (liver) or the vena cava (adipose tissue, heart, and skeletal muscle) as a bolus injection. One minute later the tissues were excised and homogenized in extraction buffer at 4 °C as described under “Experimental Procedures.” After centrifugation, aliquots containing equal amounts of protein were immunoprecipitated with anti-JAK2 antibody and protein A-Sepharose 6MB and then resolved on 6% SDS-polyacrylamide gels. The protein bands were subsequently transferred to a nitrocellulose membrane and detected with anti-phosphotyrosine antibody and 125I-protein A, after which the membrane was subjected to autoradiography. The data are representative of three experiments.

**Fig. 2.** Time course and dose-response of insulin-stimulated JAK2 tyrosine phosphorylation in rat liver (panel A) and heart (panel B). Rats were anesthetized, and the abdominal wall was incised to expose viscera. Saline or insulin (at the time and dose indicated) were administered into portal vein or vena cava as a bolus injection. In the dose-response experiment, 1 and 3 min after insulin infusions the liver and the heart, respectively, were excised and homogenized in extraction buffer at 4 °C, immunoprecipitated with anti-JAK2 or anti-insulin receptor antibodies, and immunoblotted as described in legend for Fig. 1. The data are representative of three experiments.
Insulin Induces JAK2 Tyrosine Phosphorylation

To determine whether JAK2 kinase activity was stimulated by insulin, we measured enzyme autophosphorylation in vitro using two approaches (Fig. 3, B and C). In the first approach, the liver extract was not exposed to insulin, but ATP was added to the in vitro autophosphorylation reaction. Lane 2, the liver extract was not exposed to insulin, but ATP was added to the in vitro autophosphorylation reaction. Lane 3, insulin (20 ng) was infused into the portal vein, and the liver then extracted, but no exogenous ATP was added during the in vitro autophosphorylation step. The small signal seen in this lane probably represents JAK2 autophosphorylation using endogenous ATP.

In the heart, the time course of insulin-induced JAK2 phosphorylation was similar to the liver, with a rapid increase at 1 min although the maximal increase was observed 3 min after insulin injection, followed by a decline over the 15 min of the experiment (Fig. 2B). The behavior of cardiac insulin receptor phosphorylation was also similar to the liver. The time courses of JAK2 phosphorylation in skeletal muscle and adipose tissue were similar to those of liver and heart (data not shown).

The insulin-stimulated phosphorylation of JAK2, as determined by anti-JAK2 immunoprecipitates of liver extracts, was dose-dependent (Fig. 2A). The presence of phosphorylated JAK2 was detectable after the injection of as little as 20 ng of insulin, and half-maximal stimulation occurred with 200–400 ng of the hormone (Fig. 2A). Although we could not determine portal insulin levels, in preliminary experiments peripheral insulin levels obtained 90 s after an intraportal injection of 400 ng of insulin ranged between 40 and 70 microunits/ml and were similar to the normal physiological postprandial range in rats. Maximal stimulation was observed with 2 µg of insulin and was 7–12 times greater than the basal levels (n = 3).

The dose-response relationships for the insulin stimulation of JAK2 phosphorylation from adipose tissue, skeletal muscle, and heart were similar, when insulin was infused into the portal vein. Fig. 2B shows such a relationship for cardiac tissue and indicates that the maximal effect was observed at 2 µg of insulin and decreased thereafter.

Because insulin may affect other mediators in vivo to induce these responses, we performed experiments in which the effects of insulin were analyzed in isolated adipocytes. The results are presented in Fig. 3A. A clear stimulation of JAK2 tyrosine phosphorylation was induced by insulin, showing that insulin has a direct effect on this pathway.

To determine whether JAK2 kinase activity was stimulated by insulin, we measured enzyme autophosphorylation in vitro using two approaches (Fig. 3, B and C). In the first approach,
we immunoprecipitated liver extracts (with or without insulin treatment) with anti-JAK2 antibodies and performed an in vitro kinase assay using \([\gamma^{32}P]ATP\) as described above. The results are presented in Fig. 3B and show that the JAK2 kinase activity was significantly increased in liver extracts after a portal infusion of insulin. In the second approach, a low dose of insulin (20 ng) was infused into the portal vein to obtain limited tyrosine phosphorylation of JAK2, which was then immunoprecipitated and reacted with ATP to permit autophosphorylation. The phosphorylation of tyrosine was quantified by Western blot analysis using an anti-phosphotyrosine antibody, which showed that insulin induces JAK2 autophosphorylation (Fig. 3C, lane 4). The small signal seen in the liver extract previously infused with a low dose of insulin but with no exogenous ATP added during the in vitro autophosphorylation step (Fig. 3C, lane 3) probably represents JAK2 autophosphorylation using endogenous ATP.

The rapid induction of JAK2 tyrosine phosphorylation by insulin suggests that JAK2 may associate with the insulin receptor. To test this possibility, liver extracts were immunoprecipitated with the anti-JAK2 antibody before and after insulin stimulation, and the precipitated proteins were probed with anti-receptor antibody. The experiments show that JAK2 co-precipitates with insulin receptor. Although small amounts of insulin receptor were bound to JAK2 before insulin stimulation, the addition of the hormone increased the insulin receptor-JAK2 association within 1 min. Anti-insulin receptor co-immunoprecipitation was also performed with the same tissue extracts. As shown in Fig. 4, JAK2 protein was co-immunoprecipitated by anti-insulin receptor antibody, with much higher affinity following insulin stimulation. These results were also reproduced in rat skeletal muscle, adipose tissue, and cardiac tissue and demonstrate that JAK2 physically associates with the insulin receptor clearly upon insulin stimulation.

Co-immunoprecipitation between JAK2 and IRS-1 in liver was also observed. In immunoprecipitates of IRS-1 that were blotted with anti-JAK2 antibody, a band corresponding to JAK2 was evident after insulin stimulation (Fig. 4). When the liver extracts were immunoprecipitated with anti-JAK2 antibody and blotted with anti-IRS-1 antibody, a band correspond-
Insulin Induces JAK2 Tyrosine Phosphorylation

In various cells and tissues, JAK activation has been proposed to be the signaling pathway that mediates the transcriptional activation of early growth response genes by cell surface cytokines (41). The JAK2 pathway may play a similar role in the control of insulin-induced cell growth. The shared use of JAK2 by multiple receptors is likely to reveal important connections between various hormones and cytokines that were previously unrecognized or that had been really unexplained.

Acknowledgments—We thank L. J. aneri and C. Silva for technical assistance.

REFERENCES

1. White, M. F., Maron, R., and Kahn, C. R. (1985) Nature 318, 183–186
2. Kadokawa, T., Koyasu, S., Nishida, E., Tobe, K., Izumi, T., Takaku, F., Sakai, H., Yahara, I., and Katsuya, M. (1987) J. Biol. Chem. 262, 7542–7550
3. Rothenberg, P. L., Lane, W. S., Kariuki, A., Backer, J., White, M. F., and Kahn, C. R. (1991) J. Biol. Chem. 266, 8302–8311
4. Keller, S. R., Kitajawa, K., Aebersold, R., Lienhard, G. E., and Garner, C. W. (1991) J. Biol. Chem. 266, 12817–12820
5. Sun J., Rothenberg, P. L., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) Nature 352, 73–77
6. Nishiya, M., and Wands, J. R. (1992) Biochem. Biophys. Res. Commun. 183, 280–285
7. Araki, E., Sun, J. X., Haag, B. L., Chuang, L. M., Yang-Feng, T., White, M. F., and Kahn, C. R. (1993) Diabetes 42, 1041–1054
8. Keller, S. R., Aebersold, R., Garner, C. W., and Lienhard, G. E. (1993) Biochem. Biophys. Acta 1172, 323–326
9. Sun J. X., Wang, L. M., Zhang, Y., Yenush, L., Myers, M. G. J., Glassoe, E., and White, M. F. (1995) Nature 377, 173–177
10. Prong, J. G., Michel, J., Pellicci, G., Kawasaki, K., and Poole, J. L. (1993) J. Biol. Chem. 268, 5748–5753
11. Kovacina, K. S., and Roth, R. A. (1993) Biochem. Biophys. Res. Commun. 193, 1303–1311
12. Okada, S., Yamauchi, K., and Pessin, J. E. (1995) J. Biol. Chem. 270, 20737–20741
13. Sknider, E. Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. Jr., Backer, J. M., Ulrich, A., White, M. F., and Schlessinger, J. (1993) EMBO J. 12, 1926–1936
14. Perroti, N., Accili, D., Marcus, S. B., Rees-Jones, R. W., and Taylor, S. I. (1987) J. Biol. Chem. 262, 9521–9528
15. Levy-Tolodra, R., Taouis, M., Blauet, D., Hadden, G. P., and Taylor, S. I. (1994) J. Biol. Chem. 269, 31178–31184
16. Van Horen, D. B., Myers, M. G. Jr., and Backer, J. M. (1994) J. Biol. Chem. 269, 29–32
17. Backer, J. M., Myers, M. G., Jr., Shoeston, S. E., Chin, D. J., Sun, J. X., Miralpeix, M., Hu, P., Margolis, B., Sknider, E. Y., Schlessinger, J., and White, M. F. (1992) EMBO J. 11, 3469–3479
18. Wilks, A. F., Harpur, A. G., Kurban, R. R., Rhap, S. J., Zuercher, G., and Ziehmke, A. (1993) Mol. Cell. Biol. 13, 2057–2065
19. Harpur, A. G., Andres, A. C., Ziehmke, A., Aston, R. R., and Wilks, A. F. (1992) Oncogene 7, 1347–1350
20. Silvennoinen, O., Witthuhn, B. A., Quelle, F. W., Caldwell, J. L., Yi, T., and Ihle, J. N. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8429–8433
21. Johnston, J. A., Kamamura, M., Kirken, R. A., Chen, Y. C., Blake, T. B., Shibuya, K., Ortaldo, J. R., McVicar, D. W., and O'Shea, J. J. (1994) Nature 370, 151–153
22. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T., and Ihle, J. N. (1994) Nature 370, 153–157
23. Firnbach-Kraft, L., Byers, M., Show, T., Dalla-Favera, R., and Krolewski, J. J. (1994) Oncogene 5, 1329–1336
24. Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle, J. N. (1993) Cell 74, 227–236
25. Lebrun, J. J., Ali, S., Sofer, L., Ulrich, A., and Kelly, P. A. (1994) J. Biol. Chem. 269, 14021–14026
26. Rui, H., Kirken, R. A., and Farrar, W. L. (1994) J. Biol. Chem. 269, 5364–5368
27. Argentis, L. S., Campbell, G. S., Yang, X., Witthuhn, B. A., Silvennoinen, O., Ydie, J. N., and Carter-Su, C. (1993) Cell 74, 237–244
28. Barrero, M. B., Schiffer, B., Paxton, W. G., Heerde, L. B., Berk, B. C., Delafontaine, P., and Benetsoe, E. (1993) Cell 74, 347–250
29. Saad, M. J. A., Folli, F., Kahn, J. A., and Kahn, C. R. (1993) J. Clin. Invest. 92, 1789–1794
30. Rodbell, M. (1964) J. Biol. Chem. 239, 375–380
31. Grichling, G., Levy, L. K., and Goodman, H. M. (1983) Endocrinology 113, 1111–1120
32. Laemmli, U. K. (1970) Nature 227, 680–685
33. Towbin, H., Stahl, J., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354
34. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
35. Argentis, L. S., Hsu, G. W., Myers, M. G., Billestrup, N., White, M. F., and Carter-Su, C. (1995) J. Biol. Chem. 270, 14685–14692
36. Flier, J. S. (1983) Annu. Rev. Med. 34, 145–160
37. Friedenberg, G., Klein, H. H., Cordera, R., and Olefsky, J. M. (1985) Endocrinology 111, 72, 227–236
38. Saad, M. J. A., Araki, E., Miralpeix, M., Rothenberg, P. L., White, M. F., and Kah, C. B. (1993) J. Clin. Invest. 92, 1989–1989
39. Carvalho, C. R. O., Brenelli, S. L., Silva, A. C., Nunes, A. L. B., Velloso, L. A., and Saad, M. J. A. (1996) Endocrinology 137, 151–159
40. Ihle, J. N. (1995) Nature 377, 591–594

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
Insulin Induces Tyrosine Phosphorylation of JAK2 in Insulin-sensitive Tissues of the Intact Rat
Mario J. A. Saad, Carla R. O. Carvalho, Ana C. P. Thirone and Lício A. Velloso

*J. Biol. Chem.* 1996, 271:22100-22104.
doi: 10.1074/jbc.271.36.22100

Access the most updated version of this article at [http://www.jbc.org/content/271/36/22100](http://www.jbc.org/content/271/36/22100)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/271/36/22100.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 41 references, 18 of which can be accessed free at [http://www.jbc.org/content/271/36/22100.full.html#ref-list-1](http://www.jbc.org/content/271/36/22100.full.html#ref-list-1)