

# Erythrocyte Insulin and Insulin-Like Growth Factor-I Receptor Tyrosine Kinase Activity in Hypertension in Pregnancy

James R. Sowers, David B. Jacobs, Lori Simpson, Bassam Al-Homsi, George Grunberger, and Robert Sokol

We have shown that preeclampsia is associated with insulin resistance. In the present study, we examined young normal, preeclamptic (PE), and gestational hypertensive (GH) nulliparous African-American women at term to investigate cellular determinants of this resistance and insulin and insulin-like growth factor-I (IGF-I) binding to partially purified erythrocyte receptors and receptor tyrosine kinase activity (TKA). Blood pressure was significantly elevated in PE and GH subjects as compared with controls. Insulin binding was similar in number and affinity in the three groups (femtomoles per microgram). IGF-I binding was increased in PE subjects as compared with either normals or GH subjects ( $0.2 \pm 0.02$ ,  $0.15 \pm 0.01$ , and  $0.14 \pm 0.02$  fmol/ $\mu$ g protein). Insulin receptor TKA was increased in PE subjects as compared with normals when assessed either per microgram protein or per femtomole insulin binding ( $P < .01$ ). In contrast, IGF-I-potentiated TKA was elevated in PE subjects only when assessed per microgram protein ( $P < .03$ ). Thus, the increased number of IGF-I receptors in erythrocytes of PE subjects yields a net increase in receptor tyrosine kinase. Also, there is an augmentation of insulin receptor TKA in PE subjects. Together, these two alterations may be a compensatory mechanism for the insulin resistance associated with hypertensive diseases of pregnancy.

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THERE IS CONSIDERABLE evidence that insulin resistance develops over the course of normal pregnancies.<sup>1-11</sup> Women developing hypertension during the third trimester of pregnancy appear to have an exaggerated state of insulin resistance.<sup>12</sup> Indeed, we have recently reported that women developing preeclampsia have hyperinsulinemia and elevated insulin to glucose ratios as early as the second trimester of pregnancy.<sup>13</sup> Maternal insulin resistance in all of these reports<sup>1-13</sup> is inferred from elevated insulin levels and enhanced insulin responses to a glucose load. None of these studies have explored insulin sensitivity at the cellular level.

Insulin and insulin-like growth factor-I (IGF-I) mediate their effects on cellular function through binding to specific cell-surface heterodimeric receptors consisting of two extracellular  $\alpha$ -subunits and two transmembrane  $\beta$ -subunits.<sup>14-17</sup> The  $\beta$ -subunit of the insulin receptor contains an extracellular binding domain, a transmembrane-spanning region, an intracellular adenosine triphosphate (ATP) binding region, and a tyrosine kinase domain.<sup>15-17</sup> Considerable evidence demonstrates that tyrosine kinase activity (TKA) of insulin and IGF-I receptors is essential for most actions of insulin and IGF-I.<sup>16,17</sup> Further, altered insulin receptor kinase activity has been reported in various insulin-resistant states.<sup>18-25</sup> In the present study, we examined erythrocyte insulin and IGF-I receptor TKA in nulliparous African-American women developing hypertension in pregnancy and in their gestational controls.

## SUBJECTS AND METHODS

Subjects were recruited from an ongoing longitudinal study of preeclampsia in inner-city, nulliparous African-American women<sup>25,26</sup> after provision of informed consent. Clinical determinations and blood collections were made on 27 nulliparous women at term. Blood pressures were measured in the left lateral position in triplicate at 5-minute intervals. Subjects were classified as normal ( $n = 12$ ), gestational hypertensive ([GH]  $n = 5$ ), or preeclamptic ([PE]  $n = 8$ ). Criteria used to define gestational hypertension were as follows: (1) an increase in systolic blood pressure of  $\geq 30$  mm Hg and/or an increase in diastolic blood pressure of  $\geq 15$  mm Hg above blood pressures obtained before 20 weeks' gestation, (2) a term systolic blood pressure  $\geq 140$  mm Hg and/or a diastolic blood pressure  $\geq 90$  mm Hg, and (3) proteinuria less than 1 g/L in a random collection. In addition to the above-mentioned criteria for gestational hypertension, criteria for the diagnosis of preeclampsia also included proteinuria of 1 g/L in a random collection.

### Preparation of Erythrocyte Membranes

Erythrocytes were isolated by layering a 1:2 mixture of 0.9% saline and blood onto 3 mL Ficoll-Hypaque (Pharmacia Biotechnology, Uppsala, Sweden) and centrifuged at  $1,000 \times g$ . The supernatant was aspirated and discarded. The erythrocyte pellet was again diluted, layered on Ficoll-Hypaque, and centrifuged as above. After aspirating the supernatant, the erythrocyte pellet was diluted in 5 mmol/L Tris, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1  $\mu$ g/mL aprotinin and gently stirred for 1 hour at 4°C. The cells were then centrifuged at  $35,000 \times g$  for 20 minutes. The supernatant was aspirated, and the pellet was resuspended in 0.5 mmol/L Tris, 1 mmol/L PMSF, and 1  $\mu$ g/mL aprotinin, 2  $\mu$ g/mL pepstatin, and 2  $\mu$ g/mL leupeptin, pH 7.6. Cells were then centrifuged for 20 minutes at  $35,000 \times g$ . This was repeated four times, followed by a final wash in 50 mmol/L HEPES, 1 mmol/L PMSF, and 1  $\mu$ g/mL aprotinin, 2  $\mu$ g/mL pepstatin, and 2  $\mu$ g/mL leupeptin, pH 7.6, and centrifuged at 4°C for 20 minutes at  $35,000 \times g$ . The subsequent pellet was frozen at  $-70^\circ\text{C}$  until needed for assay. Chemicals were obtained through Sigma Chemical (St Louis, MO) unless otherwise stated.

### Partial Purification of Erythrocyte Membranes

Ricin columns (E-Y Laboratories, San Mateo, CA) were preconditioned with a series of buffers (buffer I, pH 7.6: 150 mmol/L NaCl, 50 mmol/L HEPES, 0.1% Triton X-100 [Fisherbiotech, Fairlawn, NJ], and 0.01% sodium dodecyl sulfate; buffer II, pH 7.6:

From the Departments of Internal Medicine and Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI.

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Address reprint requests to James R. Sowers, MD, Professor and Director, Division of Endocrinology, Metabolism and Hypertension, University Health Center—4H, 4201 St. Antoine, Detroit, MI 48201.

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0.1 mmol/L alpha-lactose in buffer III; and buffer III, pH 7.6: 150 mmol/L NaCl, 50 mmol/L HEPES, and 0.1% Triton X-100). Ricin columns were used because they have been shown to produce an optimal yield of insulin receptors.<sup>19</sup> Erythrocyte membranes were solubilized in 50 mmol/L HEPES, 1% Triton X-100, 1 mmol/L PMSF, 1 µg/mL aprotinin, 2 µg/mL pepstatin, and 2 µg/mL leupeptin. The membranes were homogenized with a Tissumizer (Tekmar, Cincinnati, OH) and rotated for 1 hour at 4°C. The resulting solution was centrifuged for 1 hour at 190,000 × *g*. The supernatant was passed through the lectin column three times and washed with 100 mL buffer III. Membranes were eluted with buffer and collected in fractions. To inhibit proteolysis, PMSF 1 mmol/L and aprotinin 1 µg/mL were added and fractions were stored at -70°C. Protein concentration was measured by BioRad protein assay (BioRad Laboratories, Hercules, CA).

### <sup>125</sup>I-Hormone Binding

All samples were normalized for protein concentration. Hormone binding was assessed as previously described.<sup>27</sup> Briefly, samples were incubated overnight in binding buffer (150 mmol/L NaCl, 50 mmol/L HEPES, 0.1% bovine serum albumin, and 1 µg/mL aprotinin) with either <sup>125</sup>I-insulin (NEN Research Products, Wilmington, DE) or <sup>125</sup>I-IGF-I and various concentrations of unlabeled hormones (0 to 5,000 ng/mL). After incubation, samples were precipitated with 0.3% γ-globulin and 22% polyethylene glycol. Samples were then microfuged at high speed, the supernatant was removed, and the resultant pellet was washed (without vortexing) in 10% polyethylene glycol. The pellet was microfuged, the supernatant was aspirated, and the pellet was assessed using a gamma counter.

### Tyrosine Kinase

Phosphorylation of the exogenous substrate poly(Glu<sup>80</sup>Tyr<sup>20</sup>) was performed at the same time as the receptor binding assay (as previously described). Briefly, samples (60 µL) were incubated in 50 mmol/L HEPES plus 20 µL insulin or IGF-I (0 to 100 mmol/L) for 1 hour at room temperature. Forty microliters of reaction buffer (2 mg/mL poly (Glu<sup>80</sup>Tyr<sup>20</sup>), 50 mmol/L HEPES, 50 µmol/L ATP, 20 mmol/L MgCl<sub>2</sub>, 2 mmol/L MnCl<sub>2</sub>, and 1 mCi/mmol [γ-<sup>32</sup>P]ATP) was added to each sample at 30-second intervals. Filter papers (1.5 × 5 cm) were spotted with 100 µL reaction mixture at 30-second intervals 10 minutes after reaction buffer was first added. The reaction was terminated by placing the filter papers into a stopping solution (10% trichloroacetic acid and 10 mmol/L sodium pyrophosphate). Filter papers were washed three times overnight and rinsed in 95% ethanol and then acetone and dried. Radioactivity was assessed using an ICN liquid scintillation counter.

### Statistics

Comparisons of blood pressures, binding parameters, and TKA stimulation results were made by ANOVA. Results are expressed as the mean ± SEM.

## RESULTS

### Clinical Data

Mean age, body mass index, and gestational age were not significantly different for preeclamptic (PE) patients, gestational hypertensive (GH) patients, or controls (Table 1). By definition, both PE and GH patients were observed to be hypertensive, with mean arterial pressure significantly elevated as compared with control levels (controls, 84 ± 1.9 mm Hg, *n* = 12; PE, 109 ± 2.4, *n* = 8; GH, 106 ± 3.3,

**Table 1. Clinical Characteristics (mean ± SEM) of PE and GH Subjects and Controls**

Subjects	Age (yr)	Gestational Date (weeks)	MAP (mm Hg)	BMI (kg/m <sup>2</sup> )	Proteinuria (g/L)
Control (n = 12)	23 ± 1.4	35.5 ± 1.1	84 ± 1.9	31 ± 2.3	—
PE (n = 8)	26 ± 2.3	36.8 ± 0.6	109 ± 2.4*	33 ± 2.4	> 1
GH (n = 5)	27 ± 2.8	38.6 ± 0.5	106 ± 3.3*	39 ± 5.3	< 1

Abbreviations: BMI, body mass index; MAP, mean arterial pressure.

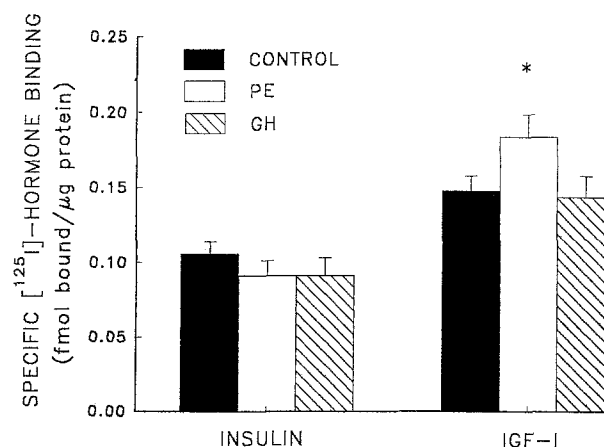
\**P* < .0001 v control.

*n* = 5), and proteinuria was only observed in PE subjects (Table 1).

### Receptor Binding

Erythrocyte insulin/IGF-I receptors were partially purified by lectin affinity chromatography. Insulin binding was similar in preparations from controls and PE and GH subjects (Fig 1). Specific <sup>125</sup>I-insulin binding was 0.1 ± 0.01 fmol/µg protein (*n* = 12), 0.091 ± 0.021 (*n* = 7), and 0.091 ± 0.021 (*n* = 5) in controls and PE and GH subjects, respectively. The half-maximal displacement of insulin binding was similar for preparations from all three groups. In contrast, <sup>125</sup>I-IGF-I binding to partially purified receptors was increased in preparations from PE subjects as compared with controls and GH subjects (Fig 1). IGF-I binding was 0.147 ± 0.008 fmol/µg (*n* = 12), 0.2 ± 0.018 (*n* = 8), and 0.143 ± 0.014 (*n* = 5) in controls and PE and GH subjects, respectively. The half-maximal displacement of insulin binding was similar in all three groups.

Scatchard plot analysis of insulin binding showed that there was no significant difference in the concentration of insulin receptors between controls and PE and GH subjects (10.3 ± 1.6, 14.1 ± 3.1, and 9.8 ± 1.2 fmol/µg protein, respectively; Fig 2A). However, there was significantly more IGF-I bound in PE subjects as compared with either controls (*P* < .05) or GH subjects (*P* < .03) (control, 17.5 ± 1.6 fmol/µg, *n* = 12; PE, 25.4 ± 3.8, *n* = 8; and GH, 15.3 ± 2.0, *n* = 5; Fig 2B).



**Fig 1. Insulin and IGF-I binding in 3 study groups. \*IGF-I binding is increased in the PE group, *P* < .05.**

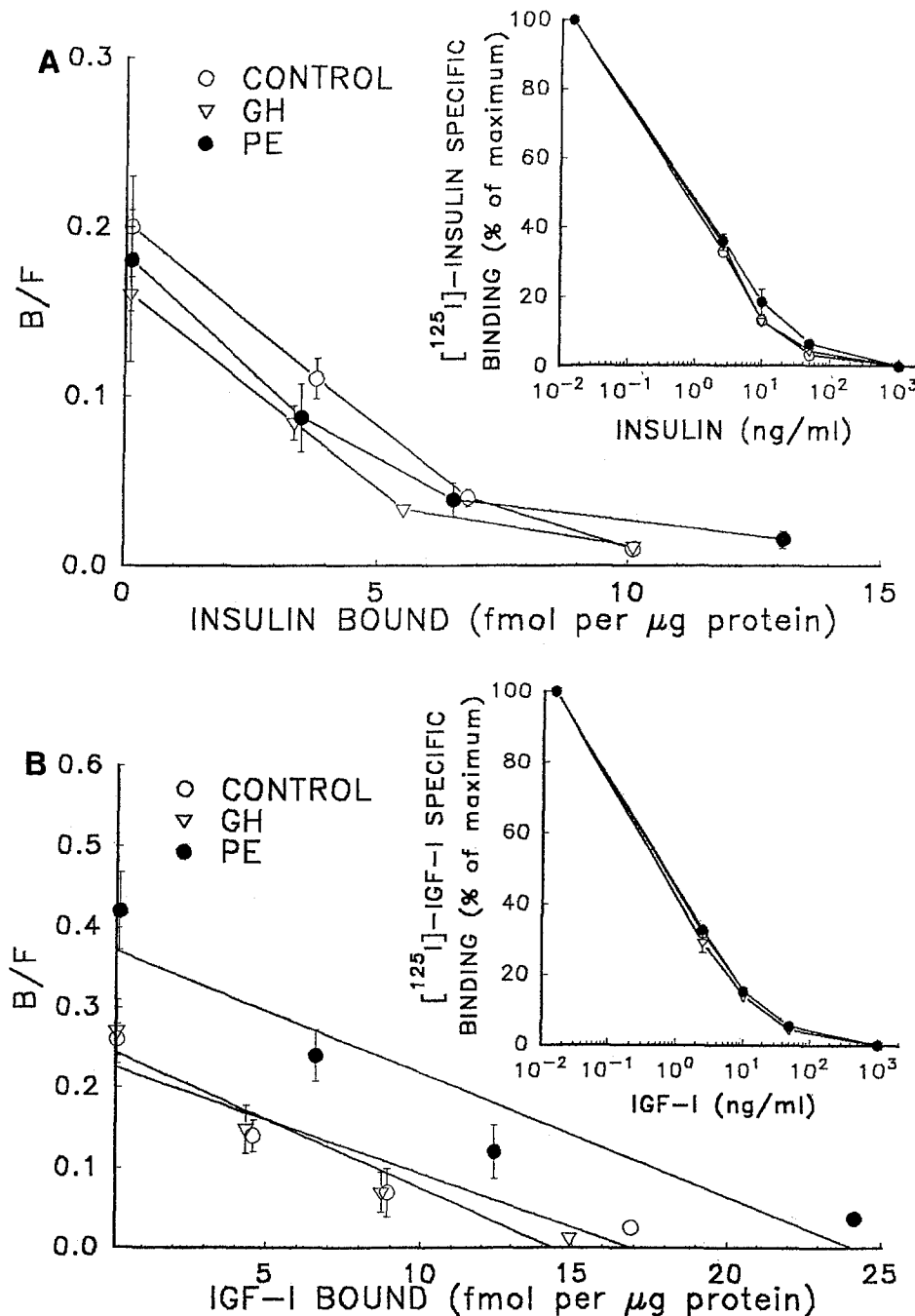


Fig 2. Scatchard plot analysis of insulin binding (A) and IGF-I binding (B) in 3 study groups. IGF-I binding is increased in the PE group,  $P < .05$ . Percent of maximal insulin and IGF-I binding is shown in the insets.

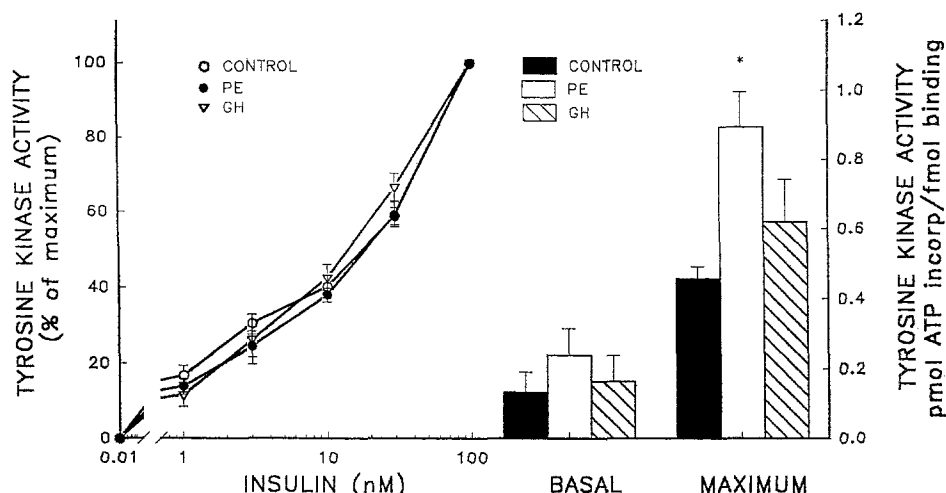
#### Receptor TKA

Insulin- and IGF-I-potentiated TKA was examined in partially purified erythrocyte receptor preparations. This was accomplished by studying insulin receptor-mediated phosphorylation of the exogenous substrate Glu<sup>80</sup>Tyr<sup>20</sup>. Insulin receptor TKA was increased in PE subjects ( $n = 8$ ) as compared with controls ( $n = 12$ ) when assessed per femtomole insulin binding ( $P < .01$ ; Fig 3B). This was also evident when insulin receptor TKA was expressed as picomoles of ATP per microgram protein ( $P < .01$ , data not shown). Insulin receptor TKA of preparations from GH subjects ( $n = 5$ ) tended to be less than that of controls

( $P = .065$ ) only when assessed per microgram protein (data not shown). There was no significant difference in half-maximal activities of insulin-potentiated TKA among the three groups (Fig 3A). IGF-I-potentiated TKA was significantly elevated in PE subjects only when assessed per microgram protein ( $P < .03$ , data not shown).

Although insulin binding was less than IGF-I binding in controls and PE subjects, insulin-potentiated (maximal-basal) TKA (picomoles of ATP per femtomole of binding) was significantly greater than that observed for IGF-I (insulin: control =  $0.3 \pm 0.02$  [ $n = 12$ ], PE =  $0.51 \pm 0.06$  [ $n = 7$ ], and GH =  $0.39 \pm 0.05$  [ $n = 5$ ]; IGF-I: con-

Fig 3. Insulin-stimulated TKA in 3 study groups. (A) Insulin-stimulated TKA as expressed per % maximal activity. (B) Basal and maximal insulin-stimulated TKA per pmol ATP incorporated per fmol insulin binding. \*TKA is increased in the PE group,  $P < .05$ .



trol =  $0.19 \pm 0.02$  [ $n = 12$ ], PE =  $0.2 \pm 0.03$  [ $n = 8$ ], and GH =  $0.21 \pm 0.02$  [ $n = 5$ ]; Fig 4).

#### DISCUSSION

Investigators have observed hyperinsulinemia and elevated insulin to glucose ratios, inferring maternal insulin resistance in the second<sup>25</sup> and third<sup>12</sup> trimesters of pregnancies associated with hypertension. Recently, several laboratories<sup>28,29</sup> have demonstrated that elevated postprandial insulin and glucose levels in the second trimester of pregnancy predict development of gestational (nonproteinuric) hypertension. We have also observed that subtle abnormalities in insulin sensitivity exist postpartum in preeclampsia.<sup>30</sup> The current study indicates that there is altered IGF-I and insulin action in erythrocytes of PE patients. Indeed, despite normal insulin binding (receptor

numbers and affinity), there was a significant increase in insulin-stimulated TKA in PE subjects. This observation is different from prior results in patients with type II diabetes, in whom insulin resistance has been associated with decreased kinase activity of insulin receptors.<sup>18-21</sup> However, our observations of increased insulin-stimulated TKA in erythrocytes from GH patients is consistent with a recent report of increased activity in individuals with type II diabetes.<sup>22</sup> Thus, the insulin resistance associated with hypertension developing in pregnancy, especially preeclampsia, is similar at the cellular level to that previously described in some type II diabetics<sup>22</sup> but not others.<sup>18-21</sup> Results of another recent study<sup>22</sup> suggest that decreased kinase activity may reflect receptor abnormalities, and increased kinase activity a postreceptor abnormality.<sup>22</sup>

Our results indicate that the initial steps of insulin action, ie, insulin binding and TKA of erythrocyte insulin receptors, are not decreased in preeclampsia, suggesting that insulin resistance in this disorder may be at the postkinase step, perhaps reflecting altered mobilization of glucose transporters. Indeed, increased insulin-stimulated hepatic TKA has also been observed in dietary rat models of insulin resistance<sup>21</sup> and in the hypertensive, insulin-resistant Zucker obese rat.<sup>23,24,31,32</sup> In the Zucker obese rat, a functional abnormality of glucose transporters (GLUT 4) in skeletal muscle has been reported.<sup>33</sup> An abnormality involving glucose transporters of erythrocytes and other tissues<sup>34</sup> may also be present in PE subjects, which is also characterized by hypertension and insulin resistance.<sup>13,26</sup> However, since the erythrocyte is not a classic insulin-sensitive cell, our results only indirectly support the concept of insulin resistance in preeclampsia.

Increased IGF-I binding and TKA was also observed in erythrocyte preparations from PE subjects. IGF-I is structurally related to insulin, and both hormones mediate their actions through highly homologous receptors with similar postreceptor phosphorylation events.<sup>14</sup> Moreover, hybrid receptors composed of insulin receptors linked to IGF receptor dimers have been identified.<sup>35</sup> These observations suggest receptor cross-reactivity, with each hormone having the potential to act through its heterologous receptor.<sup>14,35</sup>

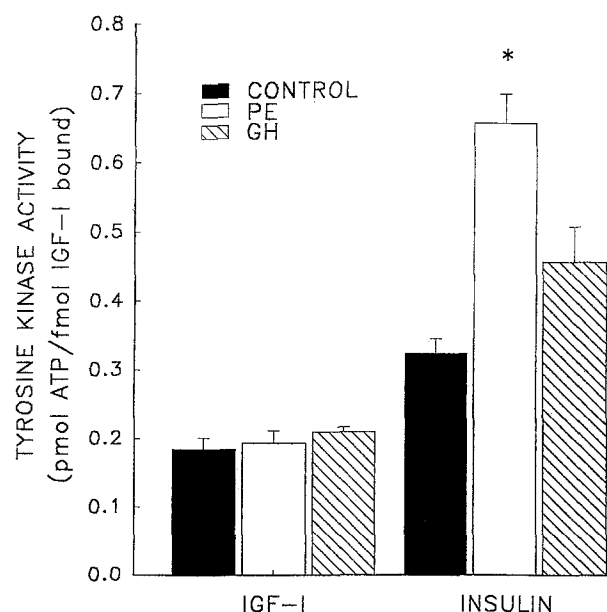


Fig 4. Incremental (maximal-basal) IGF-I- and insulin-stimulated TKA in 3 study groups. \*Increased insulin-stimulated activity in the PE group,  $P < .01$ .

Many of the tissue-proliferative effects are mediated by IGF-I in both maternal<sup>36</sup> and fetal<sup>37-41</sup> tissues. Previous studies have suggested that increased IGF-I binding and TKA in PE subjects may have relevance with respect to abnormal placental<sup>39</sup> and fetal<sup>37,38,40</sup> growth and development in this disease state. In those studies, elevated insulin-stimulated TKA was associated with elevated fasting insulin levels,<sup>21,23,24</sup> as has been observed in PE patients with associated insulin resistance.<sup>12,26</sup>

The technique used to evaluate hormone action in this cross-sectional study is limited by the fact that it does not

address possible alterations of insulin- and IGF-I-stimulated protein tyrosine phosphatase activity,<sup>42</sup> as noted previously in diabetes and pregnancy.<sup>43</sup> Indeed, the evaluation in this study of erythrocyte membrane TKA only measures net phosphate incorporation into the artificial substrate (ie, the difference between tyrosine kinase and tyrosine phosphatase(s) activities).<sup>19,42</sup> Our study is also limited by its cross-sectional nature. It will be important in future longitudinal studies to ascertain if the abnormalities described in our hypertensive-pregnancy population persist postpartum.

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