Evidence for a Direct Effect of Captopril on Early Steps of Insulin Action in BC3H-1 Myocytes

Regina S. Moisés, Carla R.O. Carvalho, Debora Shiota, and Mario J.A. Saad

Captopril, an angiotensin-converting enzyme (ACE) inhibitor, has been reported to improve insulin sensitivity. However, despite extensive investigation, the mechanisms responsible for this effect are not fully understood. Reduction of plasma angiotensin II and inhibition of kininase II have been suggested to contribute to improve insulin sensitivity. Insulin binding was measured at tracer insulin concentration in intact cells with or without captopril treatment. Specific binding, expressed as percent of total insulin added, was not different in control and captopril-treated cells. However, captopril treatment caused an increase in insulin-induced insulin receptor substrate-1 (IRS-1) phosphorylation accompanied by an increased association of IRS-1 with phosphoinositide-3 kinase (PI-3 kinase), despite no change on insulin receptor (IR) autophosphorylation. There was also an increased threonine kinase B (AKT) phosphorylation in captopril-treated cells followed by enhanced basal and insulin-stimulated glucose uptake. These results indicate that captopril treatment has a direct effect on early phosphorylation events induced by insulin in BC3H-1 myocytes.

ANGIOTENSIN-CONVERTING enzyme (ACE) inhibitors are reported to improve insulin sensitivity in several animal model[1-3] and clinical investigations.[4-6] However, despite these several studies, the mechanism by which these agents improve the action of insulin have not been fully elucidated. One putative mechanism is an inhibition of kininase II, the enzyme responsible for degradation of bradykinin. Thus, accumulation of bradykinin, or one of its metabolites such as prostaglandins, may be responsible for enhancing insulin action and insulin signaling at the skeletal muscle level.[7-10] We showed previously that bradykinin increases insulin-induced receptor and insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and association with phosphoinositide-3 kinase (PI-3 kinase) in the liver and muscle of an animal model of insulin resistance.[11] In addition, hepatic glucose production is reduced by bradykinin administration in man.[12,13] Another possibility is the hemodynamic effect of ACE inhibitors, vasodilatation, which may lead to improved capillary blood flow and increased delivery of insulin and glucose to muscle.[14-16]

These previous reports provided valuable information about the systemic effects of ACE inhibitors on insulin sensitivity; however, they did not differentiate between primary and secondary effects. Therefore, the purpose of the present study was to determine whether the ACE inhibitor captopril has a direct effect, independent of other hormonal or metabolic alterations, on insulin action. BC3H-1 myocytes, a cell line widely studied as a model for insulin action in muscle, were treated with captopril and insulin binding, insulin receptor (IR), IRS-1, and IRS-2 phosphorylation, IRS-1 and IRS-2 associations with PI-3 kinase, threonine kinase B (AKT) phosphorylation, and glucose uptake were examined.

MATERIALS AND METHODS

Materials

The reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad Laboratories (Richmond, CA). Tris, phenylmethylsulfonil fluoride (PMSF), aprotinin, dithiothreiotol, Triton X-100, Tween 20, and glycerol were obtained from Sigma Chemical Co (St Louis, MO). Protein A Sepharose 6 MB, [^{125}I] protein A, A14 mono [^{125}I] iodoinsulin, and nitrocellulose paper were from Amerscham-Pharmacia (Aylesbury, UK). Antibodies against phosphotyrosine, β-subunit IR, IRS-1, and IRS-2 were from Santa Cruz Technology (Santa Cruz, CA); antibody against the p85 subunit of PI-3 kinase was from Upstate Biotechnology (Lake Placid, NY), and antiphosphoserine-AKT antibody was from New England BioLabs (Beverly, MA). Bristol-Myers Squibb Brazil kindly provided the salt of captopril.

Cell Culture

BC3H-1 is a continuously cultured nonfusing muscle cell line derived from a nitrosoethylurea-induced neoplasm in C_3H mouse strain and was originally established by Schubert et al.[17] The cells were grown at 37°C in a humidified atmosphere of 10% CO_2 and 90% air on 35-mm dishes in Dulbecco's modified Eagle medium (DMEM; high glucose) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine, 1 nmol/L sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were fed every 4 days and used 10 days after subculturing. At this time in culture, the cells were confluent and insulin receptor binding and glucose transport rates were maximal.

Captopril Treatment of BC3H-1 Cells

Intact cells were treated with 10^{-6} and 10^{-3} mol/L captopril on day 9 for 24 hours prior to assay. Cell viability after captopril treatment, evaluated by trypan blue dye exclusion, was always greater than 90%.

Insulin Receptor Binding Assay

^{125}I-insulin binding was determined as previously described[18] with slight modifications. Monolayers cells were incubated in 1 ml of DMEM containing 20 mmol/L HEPES and 1% bovine serum albumin (BSA; pH 7.9) with 0.6 ng/mL ^{125}I-insulin at 16°C for 3 hours. ^{125}I-insulin binding was performed at 16°C because insulin internalization is negligible at this temperature[19]; thus, cell-associated ^{125}I-

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insulin essentially reflects binding only to cell surface receptors. Nonspecific binding was measured in the presence of 10 μg/mL of unlabeled human insulin that was subtracted from the total binding to reflect specific ^{125}I-binding. The binding assay was terminated by aspirating off the media, and the monolayers were washed 5 times with 1 mL of ice-cold phosphate-buffered saline (PBS) containing 1% BSA (pH 7.4). The cells were then scraped from the dishes and cell-associated radioactivity was measured in a gamma counter. Specific binding at each data point was determined in triplicate.

Protein Analysis by Immunoblotting

The assay was initiated by adding growth media with or without 10^{-7} mol/L insulin and aspirating off after 1 minute. Ice-cold extraction buffer containing 100 mmol/L Tris (pH 7.4), 10 mmol/L EDTA, 1% Triton-X-100, 100 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 10 mmol/L sodium vanadate, 2 mmol/L PMSF, and 0.01 mg aprotinin/mL was added to the dishes and the cells were scraped and collected in a Eppendorf tube. The extracts were centrifuged at 15,000 rpm at 4°C for 15 minutes to remove insoluble material; the supernatant was then used for the assay. Protein determination was performed by the Bradford dye binding method using the Bio-Rad reagent and BSA as the standard. The supernatant was used for immunoprecipitation with anti-IR, anti–IRS-1 or anti–IRS-2, and protein A-Sepharose 6MB before Laemmli sample buffer treatment and electrophoresis in SDS-PAGE as described elsewhere.[11] For whole-tissue extracts, similar sized aliquots (100 μg protein) were subjected to SDS-PAGE and immunobloted with antiphosphotyrosine antibody. Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 minutes at 120 V (constant).[20] To reduce nonspecific protein binding to the nitrocellulose, the filter was preincubated overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20). The nitrocellulose blots were incubated for 4 hours at 22°C with antibodies against phosphotyrosine, the p85 subunit of PI-3 kinase, and phosphoserine-AKT diluted in blocking buffer with 3% nonfat dry milk followed by washing for 30 minutes in blocking buffer without milk. The blots were then incubated with 2 μCi [^{125}I] protein A (30 μCi/μg) in 10 mL blocking buffer for 2 hours at room temperature and then washed again for 30 minutes as described above. [^{125}I] Protein A bound to the specific antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lightning Plus intensifying screens (Du Pont, Wilmington, DE) at −80°C for 12 to 48 hours. Band intensities were measured by optical densitometry (model GS 300, Hoefer Scientific Instruments, San Francisco, CA) of the developed autoradiographs.

Glucose Transport Assay

The rate of hexose transport was determined by measuring the initial uptake of 2-deoxy-D-[^{3}H] glucose as described in a previous publication.[21] After washing, cells were incubated in transport buffer consisting of 20 mmol/L HEPES, 120 mmol/L NaCl, 1.2 mmol/L $MgSO_4$, 2 mmol/L $CaCl_2$, 2.5 mmol/L KCl, 1 mmol/L NaH_2pO_4 (pH 7.6), with and without insulin (100 ng/mL) for 60 minutes at 37°C. The transport reaction was initiated by the addition of ^{3}H-deoxy-D-glucose (0.4 μCi) and unlabeled 2-deoxy-D-glucose (0.1 mmol/L). After 3 minutes, transport was terminated by removal of the reaction medium and washing each monolayer 8 times with ice-cold transport buffer containing 0.3 mmol/L phloretin. Cell-associated radioactivity was determined by solubilizing the cells in 1N NaOH at 50°C for 2 hours, neutralizing with HCl, and then counting in a scintillation counter. Glucose uptake at each point was the mean of triplicate determinations.

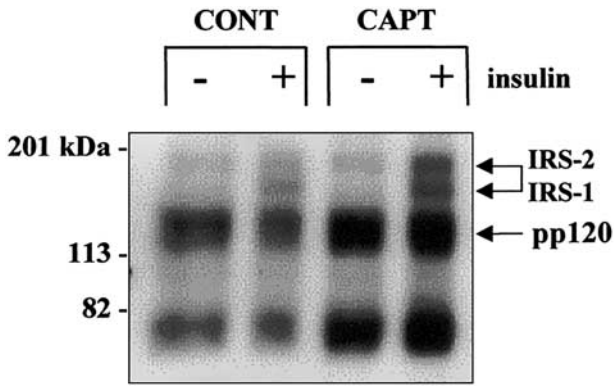

Fig 1. Effect of captopril on tyrosine phosphorylation of whole-tissue extracts. Representative blot of captopril-induced augment of tyrosine phosphorylation in BC3H-1 cell from 3 distinct experiments. Myocytes were incubated in the absence (CONT) or presence of 10^{-6} mol/L captopril for 24 hours (CAPT). The cell-soluble proteins were isolated as described in the Methods. Growth media without (-) or with 10^{-7} mol/L insulin (+) was added and aspirated off 1 minute later. The cells were homogenized in extraction buffer, centrifuged, and submitted to SDS-PAGE followed by immunoblotting with antiphosphotyrosine antibody.

RESULTS

Effect of Captopril on Insulin Binding

IR binding was measured at a tracer insulin concentration (0.6 ng/mL) in intact cells with or without captopril treatment. The results showed that specific binding, expressed as percent of total insulin added, was not different in control and captopril-treated cells, representing about 5% of the total ligand added (10^{-6} mol/L captopril: 4.93% ± 0.7%; 10^{-3} mol/L captopril: 5.14% ± 0.8%; control: 4.83% ± 0.8; P = not significant [NS]).

Effect of Captopril on IR, IRS-1, and IRS-2 Tyrosine Phosphorylation and IRS-1 Association With PI-3 Kinase

In initial experiments, using whole-tissue extracts and performing immunobloting with antiphosphotyrosine antibody, we found that captopril increased tyrosine phosphorylation of a band corresponding to pp 185 after insulin infusion, and there was also an increase in the phosphotyrosine content of 2 other bands, pp 120 and a lower band around 80 kd (Fig 1). The pp 185 is known to contain at least 2 proteins that are well characterized: IRS-1 and IRS-2. IRS-1 is the major component of this band. Incubation of this membrane with specific antibodies indicated that the lower band of pp 185 corresponds to IRS-1 and the upper to IRS-2. Thus, in Fig 1, we indicate pp 185 as IRS-1 and IRS-2.

Insulin-induced IR autophosphorylation was similar in both control and captopril-treated cells (Fig 2).

In samples from BC3H-1 cells that were immunoprecipitated with anti–IRS-1 antibodies and immunoblotted with antiphosphotyrosine (Fig 3A), there was an increase by 56% ($P < .05$) in insulin-induced IRS-1 tyrosine phosphorylation in captopril-treated cells. There is a relatively stable high-affinity interaction between IRS-1 and the p 85-kd subunit of PI-3 kinase after insulin stimulation such that both proteins are coprecipitated by

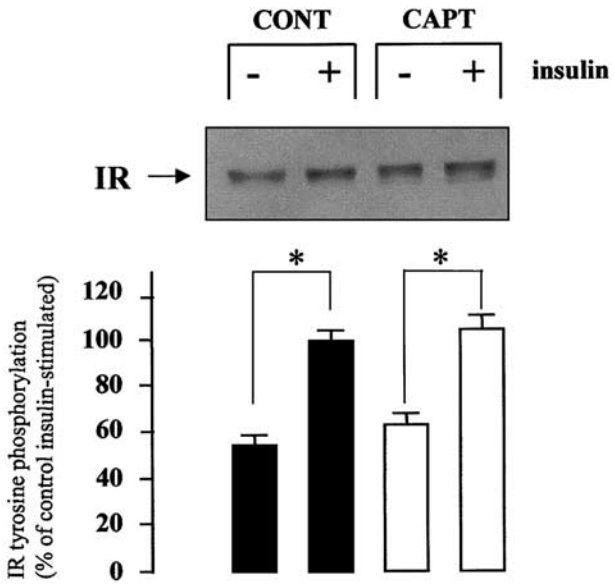

Fig 2. Effect of captopril on IR tyrosine phosphorylation. Myocytes were incubated in the absence (CONT) or presence of 10^{-6} mol/L captopril for 24 hours (CAPT). The cells were then washed and growth media without (-) or with 10^{-7} mol/L insulin (+) was added and aspirated off 1 minute later. The cells were homogenized in extraction buffer, centrifuged, and submitted to SDS-PAGE followed by immunoblotting with antiphosphotyrosine antibody as described in the Methods. Upper panel shows autoradiography from an immunoblot of a representative experiment. Bar graph shows the densitometry analysis of IR bands. Data points are means ± SEM from 3 experiments (*$P < .05$ basal *v* insulin-stimulated).

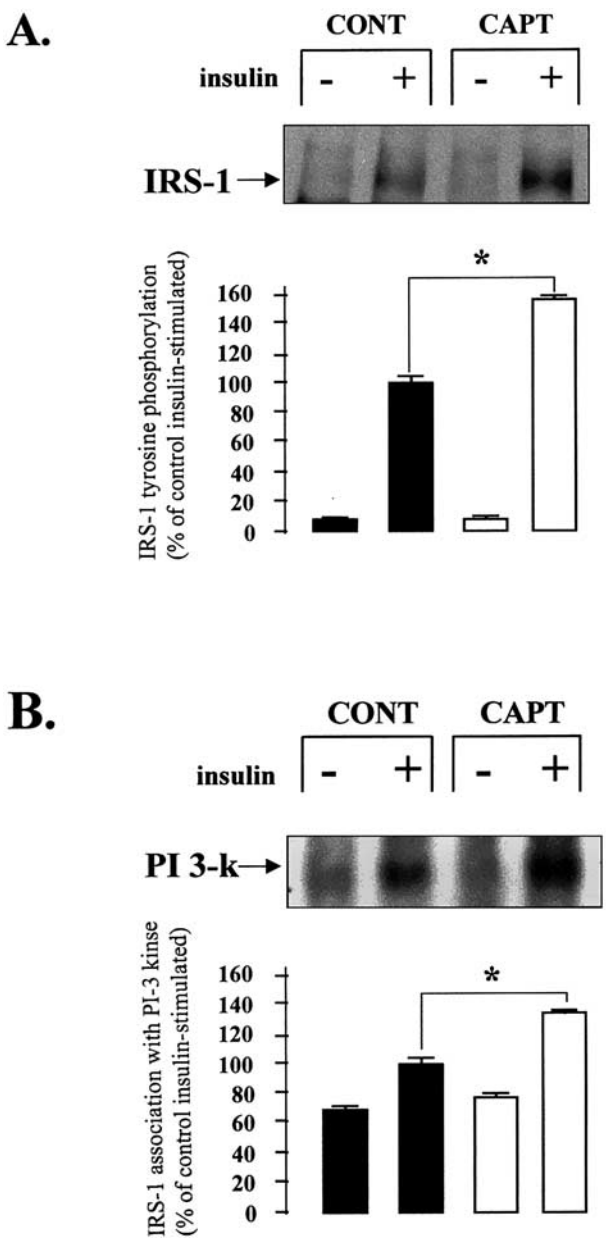

Fig 3. Effect of captopril on IRS-1 tyrosyl phosphorylation and association with PI-3 kinase. Myocytes were incubated in the absence (CONT) or presence of 10^{-6} mol/L captopril for 24 hours (CAPT). The cells were then washed and growth media without (-) or with 10^{-7} mol/L insulin (+) was added and aspirated off 1 minute later. The cells were homogenized in extraction buffer, centrifuged, and used for immunoprecipitation with anti–IRS-1 antibody as described in the Methods. The immune-complexes were submitted to SDS-PAGE followed by immunoblotting with antiphosphotyrosine antibody (A), or anti–PI-3 kinase (B). Bar graphs show the densitometry analysis of the distinct bands. Data points are means ± SEM from 3 experiments (*$P < .05$ insulin-stimulated control *v* insulin-stimulated captopril-treated cells).

antibodies against either protein.[22] Blots that had been previously immunoprecititated with anti–IRS-1 antibody were subsequently incubated with anti–PI-3 kinase antibody. Figure 3B shows that treatment of cells with captopril significantly increased insulin-stimulated PI-3 kinase association to IRS-1.

In the knockout mice for *IRS-1* gene, the downstream effects are partially compensated by the IRS-2.[23] To identify the extent of IRS-2 tyrosine phosphorylation in BC3H-1 cells treated with captopril, aliquots from the same samples were immunoprecipitated with anti–IRS-2 antibody and blotted with antiphosphotyrosine antibody. In captopril-treated cells, insulin-stimulated IRS-2 phosphorylation was similar to control BC3H-1 cells. The IRS-2 association with PI-3 kinase was also similar in both control and captopril-treated cells with no further increase after insulin stimulation (data not shown).

Effect of Captopril on AKT Phosphorylation

Stimulation of many cell types with growth factors and insulin result in activation of the PI-3 kinase/AKT.[24-26] Thus it was of interest to determine whether captopril incubation modified AKT/protein kinase B (PKB) phosphorylation induced by insulin.

Both basal and insulin-stimulated AKT phosphorylation were consistently increased in the captopril-treated cells. Also, the insulin-stimulated increase in AKT serine phosphorylation over basal was slightly higher in captopril-treated cells (Fig 4).

Effect of Captopril on Glucose Transport

We next examined the effect of captopril treatment on glucose uptake. Glucose transport was determined by measuring the initial rates of 2-deoxy-glucose uptake in control and cap-

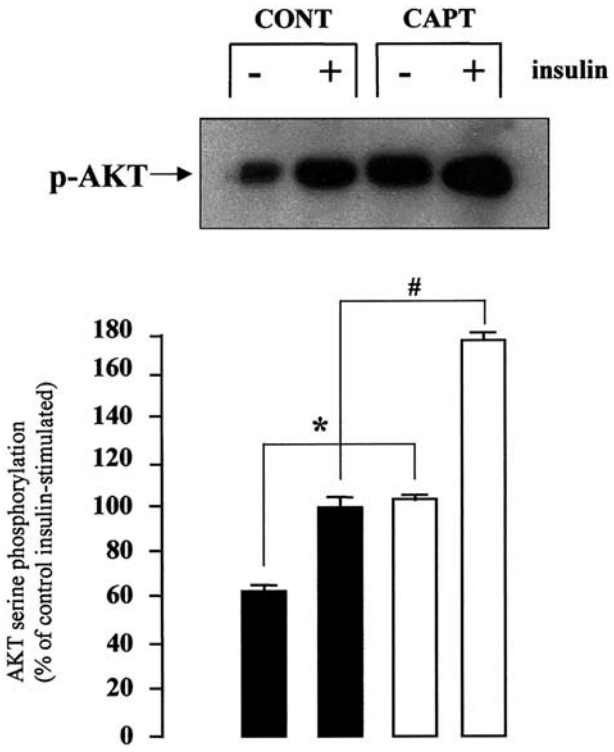

Fig 4. Effect of captopril on AKT serine phosphorylation. Myocytes were incubated in the absence (CONT) or presence of 10^{-6} mol/L captopril for 24 hours (CAPT). The cells were then washed and growth media without (-) or with 10^{-7} mol/L insulin (+) was added and aspirated off 1 minute later. The cells were homogenized in extraction buffer, centrifuged, and submitted to SDS-PAGE as described in the Methods. The immunoblotting was performed with antiphospho-AKT antibody. Bar graphs show the densitometry analysis of the distinct bands. Data points are means ± SEM from 4 experiments for each condition (*$P < .05$ basal control *v* basal captopril-treated cells; #$P < .05$ insulin-stimulated control *v* insulin-stimulated captopril-treated cells).

topril-treated cells. The results showed that treatment of cells with captopril (10^{-6} mol/L for 24 hours) increased basal glucose transport to 154% ± 15.6% ($P = .014$) and insulin-stimulated glucose transport to 217% ± 19.0% ($P = .016$) of control basal (Fig 5). The insulin-induced increase in glucose uptake was 52.4% ± 5.35% and 42.3% ± 11.5% in control and captopril-treated cells, respectively.

DISCUSSION

Several studies have suggested an improvement in insulin sensitivity in human and animal models of insulin resistance by ACE inhibitor captopril. However, the molecular mechanisms that can contribute to improve insulin action are not fully understood. In this study, we examined whether captopril exerts a direct effect on early steps of insulin action in BC3H-1 myocytes. BC3H-1 is a continuously cultured nonfusing muscle cell line derived from a neoplasm induced with nitrosoethylurea (NEU) in the C_3H mouse strain. These cells grow as undifferentiated myoblasts and develop into myocytes on reaching confluence. They possesses several structural and physiological properties of smooth muscle origin,[17] but also assume certain morphological and biochemical characteristics of skeletal muscle,[17,24-29] providing an excellent model to study the mechanism of insulin action in muscle, the most abundant insulin-sensitive tissue in the body. In addition, cultured myocyte is a suitable system to study insulin action in muscle in the absence of any hormonal, neural, or central confounding effects.

The first step in insulin action is interaction with specific receptor located on the surface of target cells. Captopril treatment of BC3H-1 myocytes had no significant effect on tracer IR binding. This is in agreement with in vivo studies where treatment with captopril did not affect specific insulin binding parameters in rats and in human studies.[30,31] The insulin binding to its receptor leads to the activation of the insulin receptor β-subunit kinase, followed by its autophosphorylation, which in turn leads to phosphorylation of adapter proteins, including members of the IRS family.[22,32-36] IRS proteins act as docking proteins for downstream signaling molecules containing Src homology 2 domains, including the 85-kd regulatory subunit of PI-3 kinase.[22] We detected that captopril treatment of BC3H-1 cells, despite no change in insulin-stimulated IR autophosphorylation, leads to increased insulin-induced IRS-1 phosphorylation accompanied by increased association with PI-3 kinase. IRS-2 signaling pathway was not altered.

There is evidence that the ability of different tissues to respond to insulin is determined by the distal effectors of insulin action present in each individual cell.[11,37,38] Using homozygous genetic disruption of *IRS-1* or *IRS-2* genes, Previs et al[39] identified an important tissue-specific role for IRS-1 and IRS-2 in mediating the effect of insulin on carbohydrate metabolism in mice in vivo. IRS-1–deficient mice have normal blood glucose concentration despite peripheral insulin resistance.[23,39] IRS-2 knockout mice in the first 6 weeks of life, with near normal glycemia, have normal rates of basal and insulin-

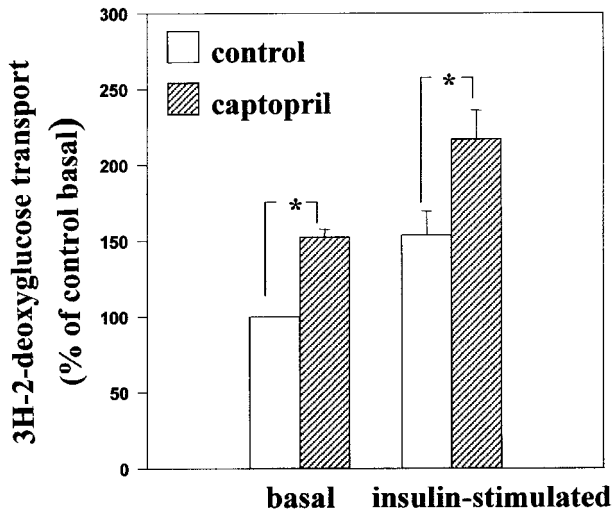

Fig 5. Effect of captopril on glucose uptake. Glucose transport was determined by measuring the initial rates of 2-deoxy-glucose uptake in control and captopril-treated cells before and after insulin stimulation. Data are mean ± SEM of 4 experiments (*$P < .05$ control *v* captopril-treated).

stimulated glucose-uptake in skeletal muscle. These findings demonstrate that IRS-2 is not necessary for glucose transport in skeletal muscle and suggest that signaling through other molecules such as IRS-1 is sufficient to mediate the effect of insulin on glucose transport in this tissue. Our results are in agreement with these data, since, captopril treatment induced an increase in basal and insulin-stimulated glucose uptake in BC3H-1 cell accompanied by an augment in insulin-induced tyrosine phosphorylation of IRS-1, with no further increase in IRS-2 phosphorylation.

Phosphorylated IRS-1 and IRS-2 can associate with PI-3 kinase, which is a key signaling transducer in insulin-mediated glucose uptake and glycogen synthesis.[26,40] The PI-3 kinase–derived phosphorylated phosphatidylinositols contribute to activation of the serine/AKT, one of the best known PI-3 kinase effectors. In BC3H-1 cells treated with captopril there was an increase in insulin-induced IRS-1/PI-3 kinase association and an AKT serine phosphorylation. It is interesting to note that even in basal condition there was also an increase in AKT serine phosphorylation in captopril-treated cells. AKT mediates a step in the activation of *GLUT1* gene expression in culture cells, GLUT 1 and GLUT4 translocation in adipose tissue and muscle, and also inactivates glycogen synthase kinase-3, resulting in activation of glycogen synthase.[40-43] Our studies showed that captopril has a direct effect on glucose uptake. This effect was observed even in the basal state and was additive to that of insulin. The mechanism underlying the effect on glucose uptake may be related to the increase in AKT serine phosphorylation, which was also observed in basal state, suggesting an effect of this drug independent of the upstream insulin signaling. The increase in IRS-1 tyrosine phosphorylation and IRS-1 association with PI-3 kinase induced by captopril may also play a role in the increased glucose uptake demonstrated with this drug.

The increased tyrosine phosphorylation of others bands besides pp 185, as detected in the whole-tissues extract, may be an important observation indicating a potential broad effect on tyrosine kinase activity or an inhibition of tyrosine phosphatase activity. However, these are possibilities yet to be adequately addressed.

We showed previously that administration of captopril to insulin-resistant aged rats augmented in liver and skeletal muscle the early steps in the insulin-signaling cascade, such as insulin-induced phosphorylation of insulin receptor and IRS-1 and the insulin-stimulated association of IRS-1 and PI-3 kinase.[11] These results are somewhat different from the present in vitro experiments, where we did find no change in insulin-stimulated IR phosphorylation. These discrepancies between studies most likely are because of the difference in the models used. Here we examined the captopril effect directly by using a cultured muscle cell line, whereas in the previous study, the whole animal was treated with captopril. In vivo, there is evidence that the increase in glucose utilization during ACE inhibitor treatment is mediated, at least in part, by hemodynamic factors such as an improved capillary blood flow and an accompanying increase delivery of insulin to the muscle.[44,45] Also, it is important to emphasize that the animal that was investigated in previous study was insulin resistant, with reduced insulin-induced receptor tyrosine phosphorylation.

To our knowledge, this is the first demonstration of captopril-induced AKT serine phosphorylation in BC3H-1 cultured cells. Since, there is no evidence in the literature indicating that these cells have the elements of the renine angiotensin system, we may suggest that our study provides evidence that captopril, a sulfydryl-containing group ACE inhibitor, has a direct effect on early steps of insulin action and also suggest a possible interaction directly with intracellular signaling molecules. These results may suggest a molecular mechanism by which this ACE inhibitor improves insulin action.

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