British Journal of Pharmacology (2002) 136, 76–80

www.nature.com/bjp

Mechanism of suppression of insulin signalling with lignocaine

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1 Lignocaine suppresses insulin-stimulated glucose transport into the cells and insulin-stimulated glycogenesis at doses equivalent to that used in the treatment of muscle pain disorder. We evaluated the direct effect of lignocaine on insulin receptor (IR) kinase activity.

2 After lignocaine (40 mM, approximately equivalent to 1%) or an equal volume (100 μl) saline had been injected into the tibialis anterior muscle of rat, insulin (50 mM g^{-1} body weight) was administered into the portal vein *in vivo*. Immunoprecipitation and immunoblotting were used to detect insulin-mediated tyrosine phosphorylation of both IR-β and insulin receptor substrate (IRS)-1, and insulin-stimulated binding of IRS-1 to p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) in the extracted muscle.

3 In the *in vitro* study, purified IR from rat liver and/or recombinant IRS-1 protein with adenosine triphosphate were incubated with lignocaine (4 or 40 mM).

4 Lignocaine reduced insulin-stimulated tyrosine phosphorylation of IR-β to $12.6 \pm 5.7\%$ ($P<0.001$), and IRS-1 to $32.1 \pm 18.8\%$ ($P<0.01$), and also reduced insulin-stimulated binding of IRS-1 to p85 to $27.4 \pm 12.7\%$ ($P<0.001$) relative to control (100%) in muscle *in vivo*.

5 The *in vitro* study revealed that lignocaine directly inhibited both basal and insulin-stimulated tyrosine phosphorylation of IR.

6 These results indicate that clinically used doses of lignocaine inhibit insulin signalling in skeletal muscle. The inhibitory effect of lignocaine on tyrosine kinase activity of the IR underlies the suppression of insulin signalling with lignocaine.

British Journal of Pharmacology (2002) **136**, 76–80

Keywords: Insulin receptor; lignocaine; local anaesthetic; phosphorylation; skeletal muscle; tyrosine kinase

Abbreviations: ATP, adenosine triphosphate; IR, insulin receptor; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis; PI3-K, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulphate

Introduction

Local anaesthetic, lignocaine (1–10 mM), is known to suppress an insulin-stimulated glucose transport or insulin-stimulated glycogenesis (Clausen *et al*., 1973; Nordenberg *et al*., 1981; Morgan *et al*., 1985; Kolaczynski *et al*., 1994; Karniel & Beitner, 2000). The precise location of the inhibitory effect of lignocaine on insulin's biological actions, however, has not been elucidated.

Kuroda *et al*. (1996; 2000) reported that local anaesthetics interact with the aromatic ring of phenylalanine (F1489) and with the negatively charged amino acids (D1487, E1492) around the sodium channel inactivation gate (DIFMTE 1487–1492). On the other hand, the insulin receptor (IR) is a tyrosine kinase receptor present on the surface of most cells. Binding of insulin to the extracellular α-subunits of IR leads to autophosphorylation of specific tyrosine residues of the intracellular activation loop of IR-β-subunit (DIYETD 1156–1161) (Hubbard, 1997). We found that there is a close resemblance of the amino acid sequences of either the aromatic or negatively charged amino acids between the sodium channel inactivation gate and the activation loop of IR. Therefore, we hypothesized that lignocaine interacts directly with the activation loop of IR and then suppresses insulin signalling.

Tyrosine autophosphorylation of IR results in activation of multiple downstream signalling pathways, leading to a diverse range of cellular responses, including glucose-transporter translocation, cell growth and proliferation, antiapoptosis, and synthesis of carbohydrates, lipids and proteins. Activated IR-β transduces the signal to downstream components by phosphorylating intracellular substrate proteins, such as insulin-receptor substrates (IRSs) and Shc (Lamothe *et al*., 1998). A further downstream key molecule of IRS-1 or IRS-2 is phosphatidylinositol 3-kinase (PI3-K), which consists of regulatory (p85) and catalytic (p110) subunits. The p85 regulatory subunit of PI3-K binds to IRS proteins, when tyrosine residues of IRSs are phosphorylated by IR-β.

To investigate the effect of lignocaine on insulin signalling, we studied changes in insulin signalling by injection of lignocaine into the rat's skeletal muscle *in vivo*, and also

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evaluated the effect of lignocaine on tyrosine phosphorylation of IR-β *in vitro*.

Methods

Materials

Anti-IR-β mouse monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-IRS-1 rabbit polyclonal antibody, anti-PI3-kinase p85 rabbit polyclonal antibody, anti-phosphotyrosine mouse monoclonal antibody (4G10), and recombinant IRS-1 protein were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.). IR from rat liver purified by affinity chromatography on wheat germ agglutinin, and chemicals, including lignocaine hydrochloride were from Sigma Chemical Co. (St Louis, MO, U.S.A.).

In vivo *analysis of the effect of lignocaine on insulin signalling at the skeletal muscle*

Adult male Sprague-Dawley rats (175–200 g) were used for this study. The study was approved by the Institutional Animal Research Committee. The rats were housed in mesh cages in a room maintained at 25°C and illuminated for 12 : 12 h cycles. The rats were provided with standard rodent chow and water *ad libitum*.

After food had been withdrawn for 18 h, the rat was anaesthetized with 70 mg kg^{-1} pentobarbitone sodium injected intraperitoneally. One hundred μl of lignocaine HCl (40 mM) dissolved in saline, or an equal volume (100 μl) of saline alone as control, was injected into the left tibialis anterior muscle. Three min after the injection of lignocaine or saline, 50 mu g^{-1} body weight of human insulin (Humulin R, Eli Lilly, Indianapolis, IN, U.S.A.) diluted with saline, or saline alone, was infused into the portal vein, as described previously (Folli *et al*., 1992; Hirose *et al*., 2000; 2001). The left tibialis anterior muscle was removed 5 min after insulin or saline injection, and frozen in liquid nitrogen.

The details of methods for detection of tyrosine phosphorylation of IR-β and IRS-1 and of detection of p85 associated with IRS-1 have been described previously (Hirose *et al*., 2000; 2001). Briefly, the frozen muscle tissue was minced with surgical scissors for 1 min and then homogenized in ice-cold lysis buffer (mM) HEPES pH 7.5 50, NaCl 150, EDTA 2, 1% (v v^{-1}) Nonidet P-40, 10% (v v^{-1}) glycerol, sodium fluoride 10, sodium vanadate 2, phenylmethylsulphonyl fluoride 1, sodium pyrophosphate 10, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1}, 0.5 μg ml^{-1} pepstatin). The homogenates were placed on ice for 30 min, and the insoluble material was removed by centrifugation at $20{,}000 \times g$ for 30 min. Aliquots of the supernatants containing equal amounts of protein, as determined using the Bradford protein assay, were subjected to immunoprecipitation for 1 h at 4°C with appropriate antibodies. Following the addition of protein A-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ, U.S.A.), the immunoprecipitates were washed three times in (mM) HEPES pH 7.5 50, with NaCl 150, EDTA 2, Nonidet P-40 0.1% (v v^{-1}), glycerol 10% (v v^{-1}), sodium fluoride 10, sodium vanadate 2, phenylmethylsulphonyl fluoride 1, sodium pyrophosphate 10, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin, and 0.5 μg ml^{-1} pepstatin. The samples were prepared for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) by adding Laemmli sample buffer and boiling for 5 min. The immunoprecipitates were subjected to SDS–PAGE in 7.5% (v v^{-1}) acrylamide solving gels and transferred electrophoretically to nitrocellulose membrane (Bio-Rad, Herculus, CA, U.S.A.). The membranes were then blocked in 5% (w v^{-1}) dried milk in PBS-Tween (phosphate-buffered saline containing 0.1% (v v^{-1}) Tween 20) for 1 h at room temperature, and then were immunoblotted with appropriate antibody. The antigen antibody complexes were visualized by chemiluminescence luminol reagent (Santa Cruz, CA, U.S.A.). Bands of interest were scanned by using MD-4000 (Alps Electric, San Jose, CA, U.S.A.) and were quantified by using National Institutes of Health Image 1.61 software (NTIS, Springfield, VA, U.S.A.).

In vitro *phosphorylation of IR and IRS-1 in the presence of lignocaine*

Purified IR (1 μg of protein) was phosphorylated with 0.2 mM of ATP for 2 min at 37°C in a 50 μl of incubation buffer (mM) HEPES 50, pH 7.4, NaCl 125, EDTA 1, $MgCl_2$ 10, $MnCl_2$ 5, dithiothreitol 5, and phenylmethylsulphonyl fluoride 1). Insulin (100 nM) and/or lignocaine (0, 4 or 40 mM) were also added before incubation. After the incubation, 0.8 μg of recombinant IRS-1 protein was added, and then the mixture was incubated for an additional 5 min (Solow *et al*., 1999). The samples were prepared for SDS–PAGE by adding Laemmli sample buffer and boiling for 5 min, followed by Western blot analysis with antiphosphotyrosine antibody.

Statistical analysis

Data were analysed by one-way analysis of variance with Bonferroni-corrected *post hoc* analysis or by unpaired *t*-test, as appropriate. The statistical significance was established at the $P<0.05$ level. All values are reported as means $\pm$ s.d.

Results

Suppression of insulin signalling in the skeletal muscle by lignocaine in vivo

To assess tyrosine phosphorylation of IR-β or IRS-1, muscle homogenates were subjected to immunoprecipitation with anti-IR-β or anti-IRS-1 antibodies followed by immunoblotting with anti-phosphotyrosine antibody. Insulin stimulation resulted in a marked increase in tyrosine phosphorylation of IR-β *in vivo* in tibialis muscles injected with saline. Injection of 40 mM of lignocaine into the tibialis anterior muscle, however, attenuated insulin-stimulated tyrosine phosphorylation of IR-β ($12.6 \pm 5.7\%$, $P<0.001$) in the muscle relative to the control (100%) (Figure 1). Consistent with the attenuation of phosphorylation of IR-β, tyrosine phosphorylation of IRS-1 following insulin treatment was also decreased ($32.1 \pm 18.8\%$, $P<0.001$) by lignocaine (Figure 2). IR and IRS-1 protein levels by Western blot analysis did not differ between the control and the lignocaine groups (Figures 1 and 2).

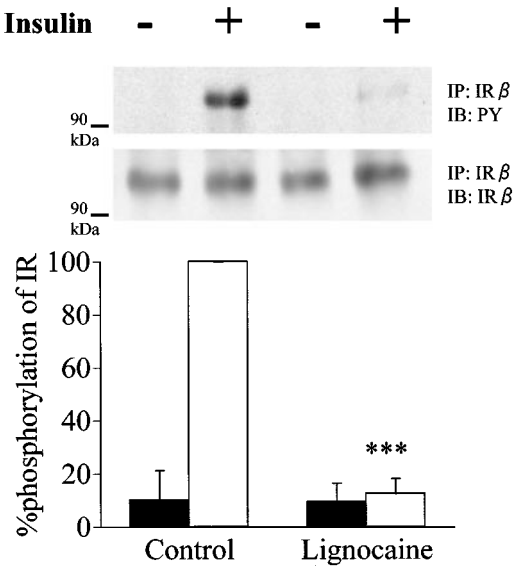

Figure 1 Levels of tyrosine phosphorylation of insulin receptor (IR) β-subunit in the left tibialis anterior muscle with lignocaine (40 mM) or saline as control. After the injection of lignocaine or saline into the tibialis anterior muscle of rat, 50 mu g^{-1} body weight of insulin was infused into the portal vein. Equal amounts of muscle protein were immunoprecipitated (IP) and immunoblotted (IB) with anti-phosphotyrosine (PY) or anti-IR-β antibody (IRβ). Solid and open bars represent basal and insulin-stimulated levels, respectively, of tyrosine phosphorylation as a percent of insulin-stimulated phosphorylation of control (100%). Insulin-stimulated tyrosine phosphorylation of IR-β (IP: IRβ, IB: PY) was attenuated by lignocaine (expressed as per cent phosphorylation normalized to IR-β protein level). IR-β protein content (IP: IRβ, IB: IRβ) was not altered by lignocaine injection. Results displayed on top two panels represent typical immunoblots of basal (insulin$-$) and insulin-stimulated (insulin$+$) phosphorylation of IR-β and immunoblots of protein content. ***$P<0.001$ vs control, $n=5$ for each lane.

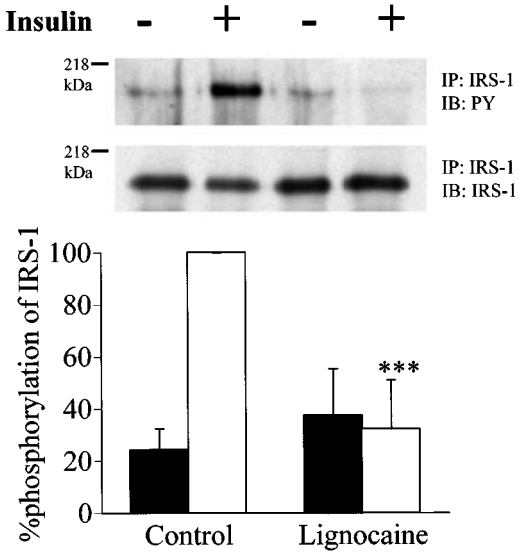

Figure 2 Levels of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) in the left tibialis anterior muscle with lignocaine (40 mM) or saline as control. After the injection of lignocaine or saline into the tibialis anterior muscle of rat, 50 mu g^{-1} body weight of insulin was infused into the portal vein. Equal amounts of muscle protein were immunoprecipitated (IP) with anti-IRS-1 antibody (IRS-1) and immunoblotted (IB) with anti-phosphotyrosine (PY). Solid and open bars on lower panel represent basal and insulin-stimulated levels, respectively, of IRS-1 phosphorylation as a percentage of insulin-stimulated phosphorylation of control (100%). Insulin-stimulated tyrosine phosphorylation of IRS-1 (IP: IRS-1, IB: PY) in muscle was attenuated by lignocaine (expressed as per cent phosphorylation normalized to IRS-1 protein level). IRS-1 protein content (IP: IRS-1, IB: IRβ) was not altered by lignocaine injection. Results displayed on top two panels represent typical immunoblots of basal (insulin$-$) and insulin-stimulated (insulin$+$) phosphorylation of IRS-1 and immunoblots of protein content. ***$P<0.01$ vs control, $n=5$ for each lane.

To further assess the consequence of the hypophosphorylation of IR, and IRS-1 on the further downstream signal transduction component, the association of IRS-1 with p85 of PI3-K was examined. The immunoprecipitates obtained with anti-IRS-1 antibody were subjected to immunoblotting with anti-PI3-K p85 antibody. In the control group, insulin stimulation resulted in a marked increase in the amount of p85 bound to IRS-1. However, this increased insulin-stimulated binding of p85 to IRS-1 was attenuated ($27.4\pm12.7\%$, $P<0.001$) in lignocaine treated animals (Figure 3).

Inhibition of insulin-stimulated tyrosine phosphorylation of IR and IRS-1 by lignocaine in vitro

To define the mechanism of inhibition of insulin signalling, lignocaine was incubated with purified IR extracted from rat liver and recombinant IRS-1 *in vitro* (Figure 4). Insulin increased autophosphorylation of IR, and also increased tyrosine phosphorylation of IRS-1. Insulin-stimulated responses of IR and IRS-1 were taken as 100%. Unstimulated basal (absence of insulin) phosphorylations of IR and IRS-1 were $53.3\pm20.7\%$ and $59.2\pm15.4\%$ of insulin-stimulated response respectively. Basal phosphorylations of IR and IRS-1 were decreased to $13.6\pm7.1\%$ and $11.5\pm2.9\%$ respectively by 40 mM of lignocaine. During insulin-stimulation, lignocaine suppressed phosphorylation of both IR and IRS-1 in a dose-dependent manner ($68.5\pm15.3\%$ and $71.4\pm23.6\%$, respectively with 4 mM of lignocaine. $16.6\pm9.0\%$ and $10.0\pm5.0\%$, respectively with 40 mM of lignocaine). These results suggest that lignocaine inhibited the basal and insulin-stimulated tyrosine kinase activity of IR and phosphorylation of IRS-1.

Discussion

Lignocaine is known to inhibit insulin-stimulated glucose transport in skeletal muscle (Clausen *et al*., 1973) and adipocytes (Kolaczynski *et al*., 1994), and also inhibits insulin-stimulated glycogenesis in hepatocytes (Morgan *et al*., 1985). Lignocaine also inhibits glycolysis in melanoma cells with suppression of cell proliferation (Karniel & Beitner, 2000). These inhibitory effects were seen at 1–10 mM concentration of lignocaine. Other local anaesthetics, such as tetracaine, procaine, and bupivacaine, also have these inhibitory effects on glucose metabolism (Clausen *et al*., 1973; Nordenberg *et al*., 1981; Morgan *et al*., 1985; Karniel & Beitner, 2000). Our present study revealed that attenuation of insulin's biological functions by lignocaine are attributable to

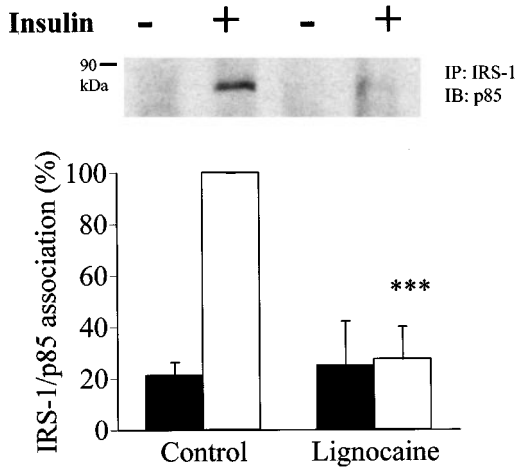

Figure 3 Insulin stimulated association of insulin receptor substrate-1 (IRS-1) with p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) in the left tibialis anterior muscle with lignocaine (40 mM) or saline as control. After the injection of lignocaine or saline into the tibialis anterior muscle of rat, 50 mu g^{-1} body weight of insulin was infused into the portal vein. Equal amounts of muscle protein, immunoprecipitated (IP) with anti-IRS-1 antibody (IRS-1), were immunoblotted (IB) with anti-p85 antibody (p85). Solid and open bars on lower panel represent basal and insulin-stimulated association of p85 with IRS-1, respectively. Insulin stimulated binding in control was taken as 100%. Lignocaine reduced the association of p85 with IRS-1. Result displayed on top panel represents typical immunoblots in the basal (insulin−) and insulin-stimulated (insulin+) state. ***$P<0.001$ vs control, $n=5$ for each lane.

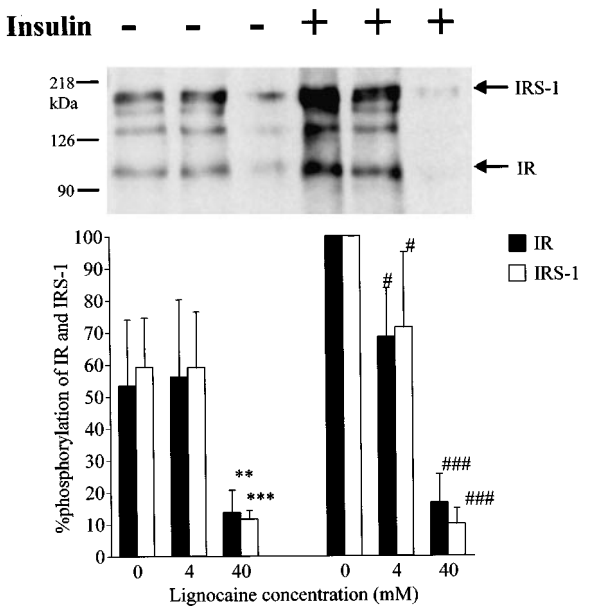

Figure 4 Phosphorylation of purified insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) in the presence or absence of lignocaine *in vitro*. Purified IR was incubated in buffer containing 0.2 mM ATP with or without 100 nM insulin, and with or without lignocaine for 2 min at 37°C, and then IRS-1 was added, followed by additional incubation for 5 min. Lignocaine reduced both basal and insulin-stimulated tyrosine phosphorylation of either IR or IRS-1. Result displayed on top panel represents typical immunoblots with (+) or without (−) insulin. **$P<0.01$, ***$P<0.001$ vs basal tyrosine phosphorylation without lignocaine, and #$P<0.05$, ###$P<0.001$ vs insulin-stimulated tyrosine phosphorylation without lignocaine, $n=4$ for each lane.

the inhibitory effect on tyrosine kinase activity of IR, which associates with decreases in IRS-1 phosphorylation and binding of IRS-1 to PI3-K, a pivotal downstream signalling molecule mediating the metabolic (anabolic) actions of insulin.

Gutiérrez-Merino & Macias (1989) pointed out that local anaesthetics are amines having aromatic rings, which can interact with aromatic amino acids, such as tyrosine and phenylalanine by cation-π interactions (Burley & Petsko, 1986; Dougherty, 1996). Kuroda *et al.* (1996; 2000) studied interactions between local anaesthetics and a sodium channel inactivation gate peptide (GGQDIFMTEEQK1484–1495 in the rat brain sodium channel IIA), which was dissected from the cytoplasmic III-IV linker connecting between domains III and IV of the sodium channel α-subunit. They concluded that the aromatic ring of local anaesthetics interacts with that of F1489 by π - π stacking (Hunter & Sanders, 1990), while its tertiary amine nitrogen interacts electrostatically with any of the negatively charged amino acids of D1487, E1492, or E1493. There are negatively charged amino acids only on both sides of the IFM residues in the III-IV linker. The amino acid sequence of the activation loop around tyrosine residues of the human IR is as follows: RDIYETDYYRK1155–1165 (Hubbard, 1997). We observe a close resemblance of the amino acid sequences between that of the sodium channel (DIFMTE1487–1492) and that of human IR (DIYETD1156–1161). Moreover, in human IR, basic amino acids such as R1155, R1164, and K1165 exist around the tyrosine residues. The positive side chains of these basic amino acids can also be an interaction site for the aromatic ring of local anaesthetics by the cation-π interactions (Dougherty, 1996). Thus it can be considered that lignocaine binds to tyrosine residues by interacting with tyrosine itself and/or with acidic (D1156, E1159, D1161) or the basic amino acids by π - π stacking, electrostatic, and cation-π interactions. Taken together, we speculate that lignocaine inhibits activity of IR kinase through the interaction with tyrosine residues themselves or residing around them in the activation loop of the IR kinase.

Local anaesthetics are used to treat myofascial pain syndrome by trigger point injection at the myofascial trigger point, which is a sensitive spot in a palpable taut band of skeletal muscle fibres (Hong, 1996). Pathophysiological changes of metabolism are known to exist at the myofascial trigger point (Hong, 1996; Gerwin, 1994). Trigger point injection is performed with 0.5–2% lignocaine concentrations approximately equivalent to 20–80 mM (Carlson *et al.*, 1993; Hong, 1994). After injection, lignocaine diffuses into the skeletal muscle to provide its therapeutic effect of relieving the muscle pain/spasm. In the *in vivo* present study, 40 mM lignocaine, which is approximately equivalent in concentration to 1% lignocaine, was administered intramuscularly. The actual amount of lignocaine injected in rat weighing 200 g was 4 μmoles (100 μl of 40 mM lignocaine), which is equivalent to 1 mmoles (25 ml of 40 mM lignocaine) in human weighing 50 kg. This calculated dose is within a clinical dose of lignocaine for the treatment of muscle pain disorder in adult humans. Therefore, lignocaine, when

injected into the muscle to treat myofascial pain syndrome, may have an adverse effect on insulin signalling at the site of injection.

In summary, lignocaine directly inhibits tyrosine kinase activity of IR in an equivalent dose of the injection into myofascial trigger point, and suppresses downstream signalling of insulin.

The authors thank Dr Masao Kaneki, Department of Anesthesia and Critical Care, Harvard Medical School, Massachusetts General Hospital, for his helpful suggestions in the preparation and writing of this manuscript. This study was supported in part by Grants-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan to Y. Tanaka, and in part by R01 Grants No. GM31569, GM55082, and GM61411 from the NIH to J.A.J. Martyn.

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(Received December 5, 2001
Revised February 18, 2002
Accepted February 21, 2002)