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Inducible nitric oxide synthase deficiency ameliorates skeletal muscle insulin resistance but does not alter unexpected lower blood glucose levels after burn injury in C57BL/6 mice

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ABSTRACT

Burn injury is associated with inflammatory responses and metabolic alterations including insulin resistance. Impaired insulin receptor substrate-1 (IRS-1)–mediated insulin signal transduction is a major component of insulin resistance in skeletal muscle following burn injury. To further investigate molecular mechanisms that underlie burn injury–induced insulin resistance, we study a role of inducible nitric oxide synthase (iNOS), a major mediator of inflammation, on burn-induced muscle insulin resistance in iNOS-deficient mice. Full-thickness third-degree burn injury comprising 12% of total body surface area was produced in wild-type and iNOS-deficient C57BL/6 mice. Insulin-stimulated activation (phosphorylation) of IR, IRS-1, and Akt was assessed by immunoblotting and immunoprecipitation. Insulin-stimulated glucose uptake by skeletal muscle was evaluated ex vivo. Burn injury caused induction of iNOS in skeletal muscle of wild-type mice. The increase of iNOS expression paralleled the increase of insulin resistance, as evidenced by decreased tyrosine phosphorylation of IR and IRS-1, IRS-1 expression, insulin-stimulated activation of phosphatidylinositol 3-kinase and Akt/PKB, and insulin-stimulated glucose uptake in mouse skeletal muscle. The absence of iNOS in genetically engineered mice significantly lessened burn injury–induced insulin resistance in skeletal muscle. In wild-type mice, insulin tolerance test revealed whole-body insulin resistance in burned mice compared with sham-burned controls. This effect was reversed by iNOS deficiency. Unexpectedly, however, blood glucose levels were depressed in both wild-type and iNOS-deficient mice after burn injury. Gene disruption of iNOS ameliorated the effect of burn on IRS-1–mediated insulin signaling in skeletal muscle of mice. These findings indicate that iNOS plays a significant role in burn injury–induced skeletal muscle insulin resistance.

Authors' contributions: Michiko Sugita: experimental design, performance of experiments, data analysis. Hiroki Sugita: experimental design, performance of experiments, data analysis. Minhye Kim: experimental design, performance of experiments, data analysis. Ji Mao: experimental design, performance of experiments, data analysis. Yoshikazu Yasuda: experimental design, performance of experiments, data analysis. Nobuyuki Shimizu: experimental design, performance of experiments, data analysis. Mayu Habiro: experimental design, performance of experiments, data analysis. Shohei Shinozaki experimental design, performance of experiments, data analysis. Shingo Yasuhara: experimental design, performance of experiments, data analysis. JAJ Jeevandra Martyn: experimental design, data analysis, editing manuscript. Masao Kaneki: experimental design, performance of experiments, data analysis, writing manuscript.

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1. Introduction

Critical illness including burn injury is associated with inflammatory response and deranged metabolism [1-3]. Despite advancements in the resuscitation and surgical treatment of burn patients, metabolic dysfunction remains a significant cause of morbidity and mortality [4]. Metabolic aberrations in critically ill patients cause muscle wasting and reduced lean body mass, which in turn lead to decreased mobility, hypoventilation, difficulties in weaning off respirators, prolonged rehabilitation and hospitalization, and even death [5-8]. A major common denominator of critical illness-associated metabolic derangements is insulin resistance, which causes protein catabolism and glycogenolysis as well as impaired insulin-stimulated glucose uptake in muscle [9,10]. The molecular bases of insulin resistance in critical illness, however, remain to be investigated.

Binding of insulin to its receptor results in activation of insulin receptor (IR) kinase, which in turn phosphorylates tyrosine residues of IR itself and insulin receptor substrates (IRSs). When phosphorylated by IR, IRSs bind and transduce the insulin signal downstream to the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). The IRSs-PI3K-Akt pathway plays a central role in the metabolic actions of insulin, including stimulation of glucose uptake, and synthesis of glycogen and protein [11]. Gene knockout of IRS-1 causes insulin resistance in skeletal muscle in mice [12], whereas gene disruption of IRS-2 induces insulin resistance in liver, but not in skeletal muscle [13]. Tissue-specific gene disruption of IRS-1 and IRS-2 also reveals that IRS-1, but not IRS-2, plays a prominent role in the metabolic actions of insulin in skeletal muscle [14-16]. Hence, impaired IRS-1–mediated insulin signaling has been recognized as a major contributor to obesity-induced insulin resistance in skeletal muscle. Consistently, we and others have demonstrated that IRS-1–mediated insulin signal transduction in response to insulin is attenuated in skeletal muscle after burn injury in rats [17-19]. However, the molecular mechanisms by which IRS-1–mediated signaling are impaired are yet to be elucidated.

Chronic inflammatory response has been implicated in the pathogenesis of obesity-related insulin resistance [20-22]. However, our knowledge remains limited about how this inflammatory response causes and/or exacerbates insulin resistance. Inducible nitric oxide synthase (iNOS) is a major mediator of inflammation. Although iNOS plays an important role in the immunological defense mechanisms against microorganisms, iNOS also exerts detrimental effects in various tissues including skeletal muscle in critical illness [23-25]. We and others have shown that iNOS plays an important role in obesity and lipopolysaccharide-induced insulin resistance in skeletal muscle and liver [26-30]. To investigate the role iNOS in burn injury–induced insulin resistance, we examined the effects of burn injury on IR/IRS-1–mediated insulin signaling in skeletal muscle of iNOS knockout (–/–) mice.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice and iNOS knockout (–/–) mice at 14 weeks of age, purchased from the Jackson Laboratory (Bar Harbor, ME), were used for this study. The iNOS–/– mice were backcrossed onto wild-type C57BL/6 mice by at least 10 generations. The study was approved by the Institutional Animal Care Committee. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were housed in a pathogen-free animal facility maintained at 25°C and illuminated by 12:12-hour light-dark cycles. The mice were provided with standard rodent chow and water ad libitum. Both wild-type and iNOS–/– mice were divided into 4 groups (sham-burn with saline or insulin, and burn with saline or insulin). Each group consisted of 8 animals, unless otherwise indicated in the figure legends. Full-thickness third-degree burn injury comprising 12% of total body surface area was produced, as described previously [31]. Briefly, mice were treated by immersing the abdomen for 8 seconds in 80°C water under anesthesia (pentobarbital sodium, 50 mg/kg body weight [BW], intraperitoneally). Buprenorphine (0.1 mg/kg BW, subcutaneously) was administered every 8 hours up to 48 hours after burn or sham-burn. Sham-burned mice were immersed in lukewarm water. Just before and at 1, 3, and 7 days after burn or sham-burn injury, the rectus abdominis muscle was excised under anesthesia for biochemical analyses. The animals were then immediately euthanized with an overdose of pentobarbital sodium (200 mg/kg BW, intraperitoneally). The study was performed in pair-fed burn and sham-burned mice. The average food intake was 1.9 g/d.

2.2. Tissue homogenization

Burned or sham-burned mice were anesthetized with pentobarbital sodium (50 mg/kg BW, intraperitoneally). Following an overnight fasting, insulin (1 or 5 U/kg BW; Humulin R, Eli Lilly, Indianapolis, IN) or saline was injected via the portal vein; and tissues were harvested at 90 seconds or 5 minutes thereafter, as indicated in the figure legends. Tissue samples were homogenized as described previously [32]. Briefly, tissues were powdered under liquid nitrogen and homogenized in ice-cold homogenization buffer (50 mmol/L HEPES-NaOH, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Nonidet P-40, 10% glycerol, 10 mmol/L sodium fluoride, 2 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium pyrophosphate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). After incubation on ice for 30 minutes, the homogenized samples were centrifuged at 13 000*g* for 30 minutes. Aliquots of the supernatant containing equal amounts of protein, determined by the Bradford protein assay, were subjected to immunoprecipitation or sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.3. Immunoblotting

Immunoblot analysis was performed as described previously [33]. Briefly, tissue homogenates containing equal amounts of protein or immunoprecipitates were subjected to 10% or 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoretic transfer to nitrocellulose membrane (Bio-Rad, Hercules, CA), the membranes were blocked in 5% nonfat dry milk for 2 hours at room temperature, followed by incubation for 2 hours at room temperature or

overnight at 4°C with anti-IR, anti-IRS-1, anti-iNOS, anti-p85 (Upstate, Lake Placid, NY), anti-phosphotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-GSK-3β (Ser 9) (Cell Signaling, Beverly, MA), anti-GSK-3β (Invitrogen, Carlsbad, CA), or anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Treviden, Gaithersburg, MD) antibody. The membranes were then incubated with anti-rabbit or anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase for 1 hour at 4°C. Western blotting chemiluminescence luminol reagent (Perkin-Elmer, Boston, MA) was used to visualize the blots. Bands of interest were scanned with the use of Power Look (UMAX Technologies, Dallas, TX) and quantified by National Institutes of Health Image 1.62 software (NTIS, Springfield, VA).

2.4. *Immunoprecipitation*

Immunoprecipitation was performed by incubating the lysates with the antibody at 4°C for 5 hours. The immune complexes were collected by incubation with protein A– or protein G–agarose beads for 1.5 hours at 4°C, washed 3 times with wash buffer B (50 mmol/L HEPES-NaOH, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.1% Nonidet P-40, 10% glycerol, 10 mmol/L sodium fluoride, 2 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium pyrophosphate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin), and boiled in Laemmli sample buffer.

2.5. *PI3K assay*

Phosphatidylinositol 3-kinase activity in the immunoprecipitates with anti-IRS-1 antibody was measured by in vitro phosphorylation assay using phosphatidylinositol (Sigma, St Louis, MO) as a substrate as described previously [33]. Briefly, 5 μL of 100 mmol/L $MgCl_2$ and 10 μL of phosphatidylinositol (2 mg/mL) sonicated in kinase buffer (10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.1 mmol/L sodium vanadate) were added to the immunoprecipitates. The PI3K reaction was initiated by the addition of 1.5 μL of 1.5 mmol/L adenosine triphosphate containing 10 μCi of [γ-^{32}P] adenosine triphosphate. After incubation for 10 minutes at 37°C, the reaction was stopped by the addition of 10 μL of 6 N HCl and 80 μL of $CHCl_3$/methanol (1:1). The samples were centrifuged, and the lower organic phase was applied to a silica gel thin-layer chromatography plate (Sigma), which had been prebaked for 1 hour. The plate was developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (100:78:19:3.3), dried, and visualized by autoradiography.

2.6. *Insulin tolerance test*

At 3 days after burn or sham-burn, following 4-hour fasting, blood glucose levels were measured just before and at 15, 30, 60, 90, and 120 minutes after insulin injection (1 U/kg BW, Humulin R). Insulin concentrations in plasma samples obtained just before the insulin injection were measured by enzyme-linked immunosorbent assay (Crystal Chem, Downers Grove, IL).

2.7. *Insulin-stimulated glucose uptake in isolated skeletal muscle*

Glucose uptake was measured as previously described [34]. Briefly, at 3 days after burn or sham-burn, mice were anesthetized following an overnight fasting; and the rectus abdominis was excised and rapidly split by mid-incision into 2 muscle strips. After the muscle strips were rinsed briefly in 5 mL Krebs-Henseleit bicarbonate (KHB) buffer supplemented with 32 mmol/L mannitol, they were incubated in 5 mL of KHB buffer supplemented with 8 mmol/L glucose and 32 mmol/L mannitol in the presence or absence of insulin (2 mU/mL, Humulin R) in a shaking water bath at 37°C for 20 minutes. The muscles were then rinsed for 10 minutes at 37°C in KHB buffer containing 36 mmol/L mannitol and 0.1% bovine serum albumin with or without insulin (2 mU/mL). Next, the muscles were incubated for 20 minutes at 37°C in 2 mL of KHB buffer containing 2-deoxy-[^{3}H] glucose (2.5 Ci/mL; PerkinElmer, Waltham, MA) and 36 mmol/L [^{14}C] mannitol (0.3 μCi/mL, PerkinElmer) with or without insulin in a shaking incubator. Buffers were gassed continuously with 95% O_2:5% CO_2 throughout the experiment. The muscles were then rinsed with KHB buffer, rapidly blotted, weighed, and solubilized by incubation at 60°C for 1 hour in 0.5 mL of 1 N NaOH. Radioactivity was counted in the sample using a scintillation counter. 2-Deoxy-[^{3}H] glucose uptake rates were corrected for extracellular trapping using [^{14}C] mannitol counts [34]. Insulin-stimulated glucose uptake was calculated based on differences in glucose uptake in the presence and absence of insulin.

2.8. *Isolation of total RNA and quantitative reverse transcriptase polymerase chain reaction*

Total RNA was isolated with an RNeasy Mini kit (Qiagen, Valencia, CA). The first-strand cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Real-time reverse transcriptase polymerase chain reaction analyses were performed as previously described [35] using 10 ng cDNA and TaqMan probes (Applied Biosystems) for mouse phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) conducted with Mastercycler ep realplex (Eppendorf, New York City, NY). mRNA expression level of mouse 36B4 [36,37] with SYBR Green (Applied Biosystems). The mRNA levels of PEPCK and G-6-Pase were normalized to those of 36B4.

2.9. *Statistical analysis*

The data were compared with one-way analysis of variance (ANOVA) followed by Fisher protected least significant differences test for the analysis of time-dependent effects of burn injury in wild-type mice. To compare the effects of burn in wild-type and iNOS knockout mice, two-way ANOVA was performed using genotype (wild-type or iNOS knockout), treatment (burn or sham-burn), and injection (insulin or saline) as individual variables. When the two-way ANOVA indicated a significant overall difference, significance

between the pairs of the groups was determined by Bonferroni post hoc multiple comparison test. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). A value of $P < .05$ was considered statistically significant. All values are expressed as means ± SEM.

3. Results

3.1. *Burn injury caused iNOS induction in association with impaired insulin signaling in wild-type mice*

First, we examined the effects of burn injury on iNOS expression in wild-type mice. Immunoblot analysis revealed robust expression of iNOS in skeletal muscle at 3 days after burn injury (Fig. 1A). Glyceraldehydes-3-phosphate dehydrogenase protein expression was not affected by burn injury, and iNOS was not detected in skeletal muscle before burn injury (Fig. 1B). Concomitant with iNOS induction, there was a prominent decrease in insulin-stimulated tyrosine phosphorylation of IR and IRS-1. This effect was most pronounced at 3 days after burn injury (Fig. 1B, C, D). Although IR expression was not altered by burn injury, IRS-1 expression was significantly reduced at 3 and 7 days after burn injury (Fig. 1D). Even after normalization to IRS-1 protein expression, insulin-stimulated phosphorylation was significantly decreased at 3 days after burn injury (Fig. 1E). These findings are consistent with our previous study in rats that showed that IRS-1–mediated signaling was impaired in skeletal muscle after burn injury [17]. Of note, burn injury–induced impairment in IRS-1–mediated insulin signaling paralleled iNOS induction in skeletal muscle in mice. As expected, iNOS induction was not observed in sham-burned wild-type controls (Fig. 2A).

3.2. *iNOS deficiency ameliorated impaired IRS-1–mediated insulin signaling in skeletal muscle of burned mice*

To investigate the role of iNOS in burn-induced insulin resistance, we examined the effects of burn injury in iNOS knockout (–/–) mice at 3 days after burn injury or sham-burn injury in comparison with wild-type mice. Inducible nitric

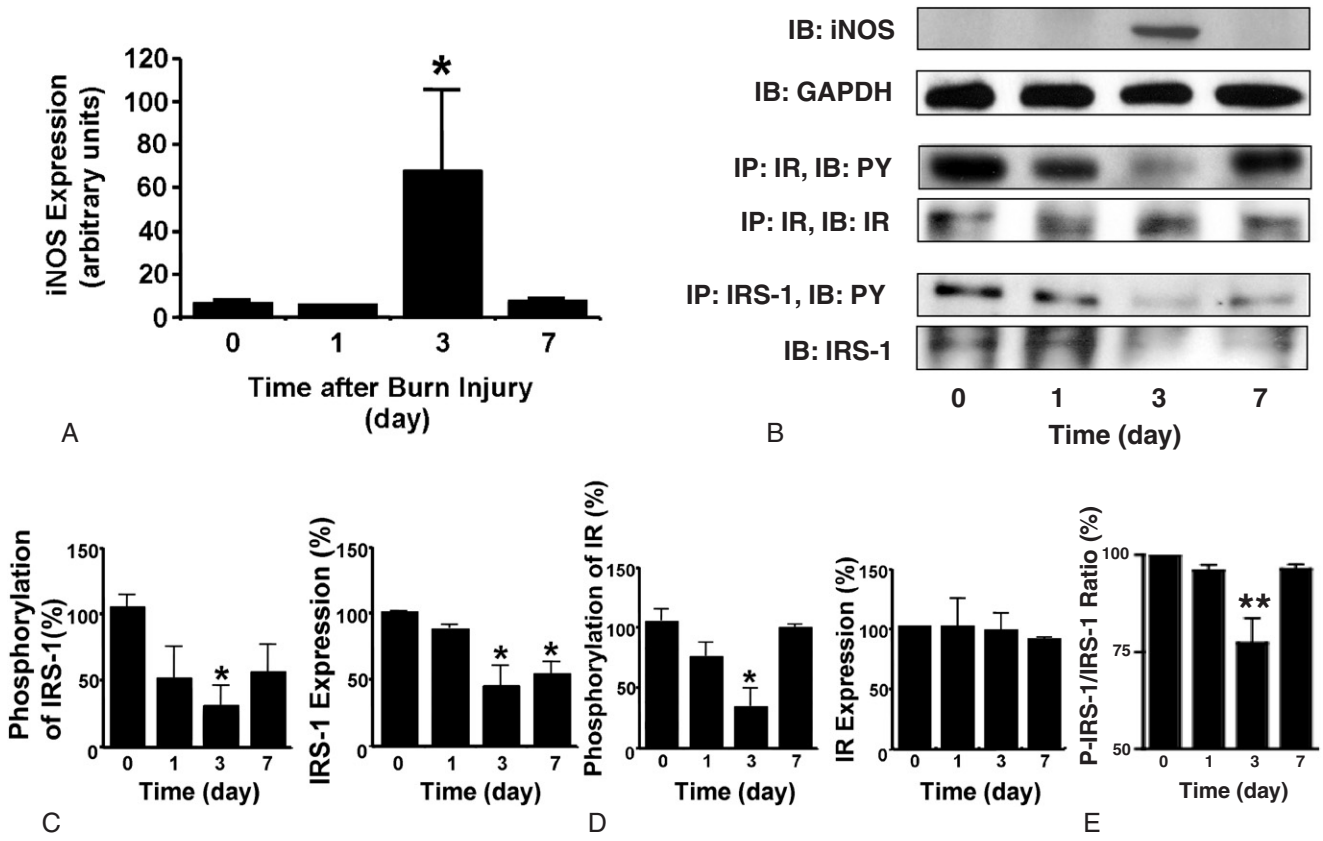

Fig. 1 – Induction of iNOS paralleled attenuated IR- and IRS-1–mediated insulin signaling in skeletal muscle after burn injury in wild-type mice. A, Immunoblotting (IB) revealed a robust induction of iNOS expression at 3 days after burn injury. The protein expression of GAPDH was not altered following burn injury. B, C, and D, Before (0) or at 1, 3, and 7 days after burn injury, insulin (5 U/kg BW) was injected; and muscle was excised 90 seconds thereafter. Immunoprecipitation (IP) followed by immunoblotting demonstrated that insulin-stimulated tyrosine phosphorylation (PY) of IR and IRS-1 was significantly decreased at 3 days after burn injury. The protein expression of IR was unaltered by burn injury. In contrast, IRS-1 protein expression was significantly decreased at 3 and 7 days after burn injury as compared with before burn injury. E, The ratio of insulin-stimulated IRS-1 phosphorylation normalized to IRS-1 protein expression was significantly decreased at 3 days after burn injury. *P < .05, **P < .01 vs before burn injury. n = 3 per group.

oxide synthase was not detected in skeletal muscle of iNOS–/– mice regardless of burn or sham-burn. Glyceraldehydes-3-phosphate dehydrogenase protein expression was unaltered by either burn injury or iNOS deficiency (Fig. 2A).

In wild-type mice, insulin injection induced robust tyrosine phosphorylation of IR in skeletal muscle of sham-burned animals, whereas insulin-stimulated tyrosine phosphorylation of IR in burned mice was decreased to 28% of that in sham-burned wild-type mice ($P < .01$) (Figs. 2B, C). Insulin-stimulated tyrosine phosphorylation of IR in burned iNOS–/– mice was 2-fold greater than that of burned wild-type mice ($P < .05$). However, insulin-stimulated phosphorylation of IR following sham-burn injury did not differ between wild-type and iNOS–/– mice. Protein expression of IR was not affected by burn injury or disruption of iNOS (Figs. 2B, C).

Insulin induced marked tyrosine phosphorylation of IRS-1 in skeletal muscle of sham-burned mice. Although insulin failed to increase tyrosine phosphorylation of IRS-1 in burned

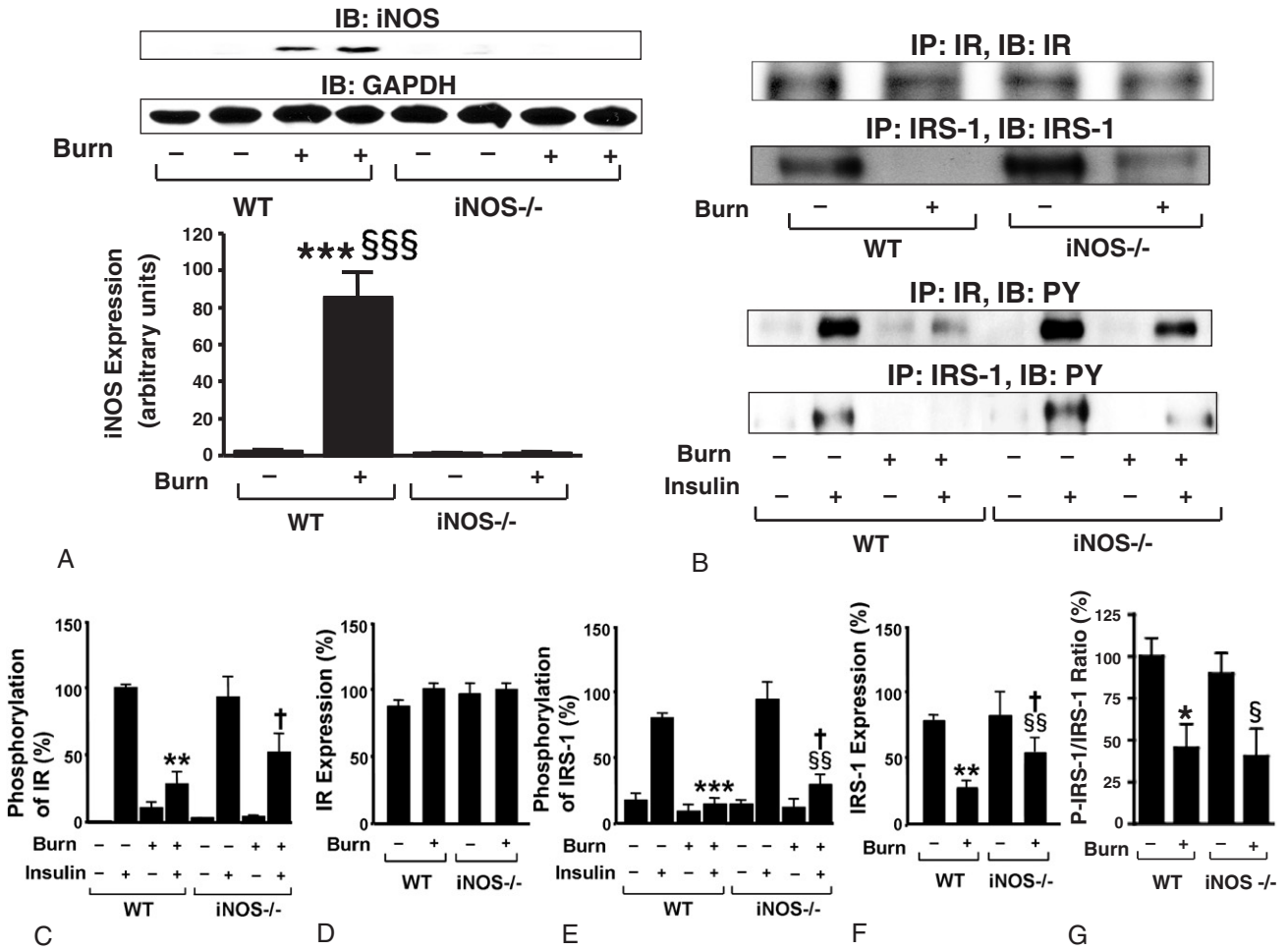

Fig. 2 – Inducible nitric oxide synthase deficiency mitigated burn injury–induced impairment in IR- and IRS-1–mediated insulin signaling in skeletal muscle. At 3 days after burn injury or sham burn, insulin (5 U/kg BW) or saline was injected; and 90 seconds thereafter, skeletal muscle was taken. A, Immunoblotting (IB) demonstrated that iNOS protein expression was induced by burn injury in wild-type, but not iNOS-deficient, mice. The protein expression of GAPDH was not affected by burn injury or iNOS deficiency. The overall interaction between genotype and burn/sham is statistically significant ($P < .001$). ***$P < .001$ vs sham-burned WT, §§§$P < .001$ vs burned iNOS –/–. B, C, D, and E, Immunoprecipitation (IP) followed by immunoblotting revealed that insulin-stimulated tyrosine phosphorylation (PY) of IR and IRS-1 was suppressed by burn injury in wild-type (WT) mice, which was ameliorated by iNOS deficiency (–/–). Neither burn injury nor iNOS deficiency altered IR protein expression. The overall interactions between genotype and other factors (burn/sham and insulin/saline) are statistically significant for phosphorylation of IR and IRS-1 ($P < .05$). **$P < .01$, ***$P < .001$ vs sham-burned WT with insulin; §§$P < .01$ vs burned iNOS –/– with insulin; †$P < .05$ vs burned WT with insulin; ‡‡$P < .01$ vs sham-burned WT with insulin. F, In contrast, IRS-1 protein expression was suppressed by burn injury. Inducible nitric oxide synthase deficiency increased IRS-1 expression in burned mice. The overall interaction between genotype and burn/sham is statistically significant ($P < .05$). **$P < .01$ vs sham-burned WT, §§$P < .01$ vs sham-burned iNOS –/–, †$P < .05$ vs burned WT. G, The ratio of insulin-stimulated IRS-1 phosphorylation (p-IRS-1) normalized to IRS-1 protein expression was significantly decreased by burn injury in wild-type and iNOS-deficient mice as compared with sham controls. This effect of burn injury on p-IRS-1/IRS-1 ratio was not altered by iNOS deficiency. *$P < .05$ vs sham-burned WT, §$P < .05$ vs sham-burned iNOS –/–.

wild-type mice, disruption of the iNOS gene partially restored tyrosine phosphorylation of IRS-1 in response to insulin in burned mice ($P < .05$) (Figs. 2B, E). In contrast to unaltered IR expression in burned mice, protein expression of IRS-1 in burned wild-type mice was decreased to 36% of that in sham-burned wild-type mice ($P < .01$). Insulin receptor substrate–1 expression in burned iNOS–/– mice was increased 2-fold compared with that in burned wild-type mice ($P < .05$) (Fig. 2B, D). When insulin-stimulated phosphorylation of IRS-1 is normalized to IRS-1 protein expression, iNOS deficiency did not significantly alter the ratio of phosphorylated IRS-1 to IRS-1 protein expression (Fig. 2E). These results suggest that the improved insulin-stimulated phosphorylation of IRS-1 by iNOS deficiency may be mainly attributable to the increased IRS-1 protein expression in burned mice. Regardless, insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1 protein expression did not differ between wild-type and iNOS–/– sham-burned mice.

We further investigated the effects of burn and disruption of iNOS on PI3K, a key downstream molecule of the insulin signaling pathway. Insulin increased p85 PI3K binding to IRS-1 in skeletal muscle of sham-burned mice. Yet, consistent with the failure of insulin to increase IRS-1 tyrosine phosphorylation in burned wild-type mice, the binding of p85 PI3K to IRS-1 did not increase in response to insulin in burned wild-type mice (Fig. 3A). Again, as with IRS-1 tyrosine phosphorylation, disruption of iNOS restored insulin-stimulated p85 PI3K binding to IRS-1 in burned mice, albeit to a lesser extent compared with that in sham-burned mice (Fig. 3B). The protein expression of p85 PI3K was unaltered by burn injury or disruption of iNOS. Consistently, the burn injury–induced decrease of insulin-stimulated PI3K activity was significantly ameliorated in iNOS–/– mice (Fig. 3C). Although insulin-stimulated PI3K activity in sham-burned iNOS–/– mice appeared to be greater than that in sham-burned wild-type mice, this was not statistically significant.

3.3. *iNOS deficiency prevented burn-induced reductions in insulin-stimulated Akt phosphorylation and ex vivo glucose uptake in mouse skeletal muscle*

Consistent with the effects of burn and iNOS deficiency on PI3K activity, insulin-stimulated phosphorylation of Akt was significantly reduced by burn injury in wild-type mice; and gene disruption of iNOS reverted this burn-induced decrease in insulin-stimulated Akt phosphorylation (Fig. 4A). Impaired insulin-stimulated activation by burn injury and its restoration by iNOS deficiency were corroborated by phosphorylation

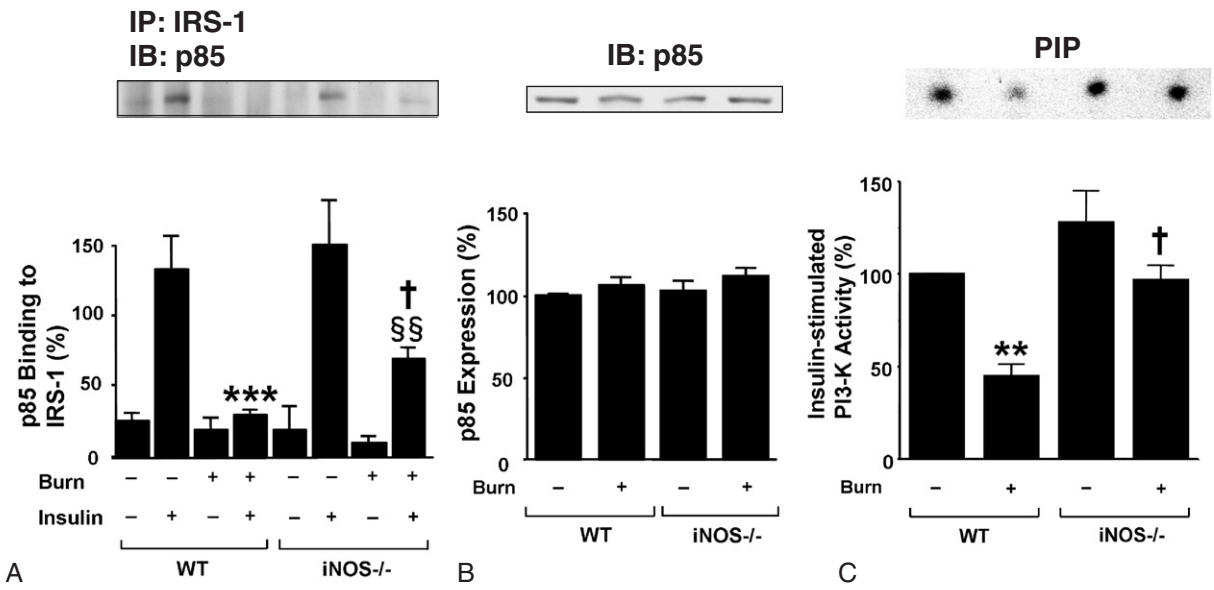

Fig. 3 – Inducible nitric oxide synthase deficiency ameliorated attenuated IRS-1–mediated PI3K activation in skeletal muscle of burned mice. At 3 days after burn injury or sham burn, insulin (5 U/kg BW) or saline was injected; and 90 seconds thereafter, skeletal muscle was taken. A and B, Insulin injection resulted in a robust increase in binding of p85 PI3K to IRS-1 in sham-burned mice, as judged by immunoprecipitation (IP) with anti-IRS-1 antibody followed by immunoblotting (IB) with anti-p85 antibody. However, insulin failed to increase p85 binding to IRS-1 in burned wild-type (WT) mice. Inducible nitric oxide synthase deficiency (–/–) significantly improved insulin-stimulated binding of p85 to IRS-1 in burned mice. The protein expression of p85 was not affected by burn injury or iNOS deficiency. The overall interaction between genotype and other factors (burn/sham and insulin/saline) is statistically significant for binding of p85 PI3K to IRS-1 ($P < .05$). ***$P < .001$ vs sham-burned WT with insulin, §§$P < .01$ vs sham-burned iNOS –/– with insulin, †$P < .05$ vs burned WT with insulin. n = 4 per group of animals with saline; n = 8 per group of animals with insulin. C, PI3K activity was evaluated in vitro phosphorylation of phosphatidylinositol (PI). Insulin-stimulated PI3K activity was decreased after burn compared with sham burn in wild-type mice. Inducible nitric oxide synthase deficiency almost completely reversed decreased PI3K activity in burned mice. The overall interaction between genotype and burn/sham is statistically significant ($P < .05$). **$P < .01$ vs sham-burned WT, †$P < .05$ vs burned WT. n = 4 per group.

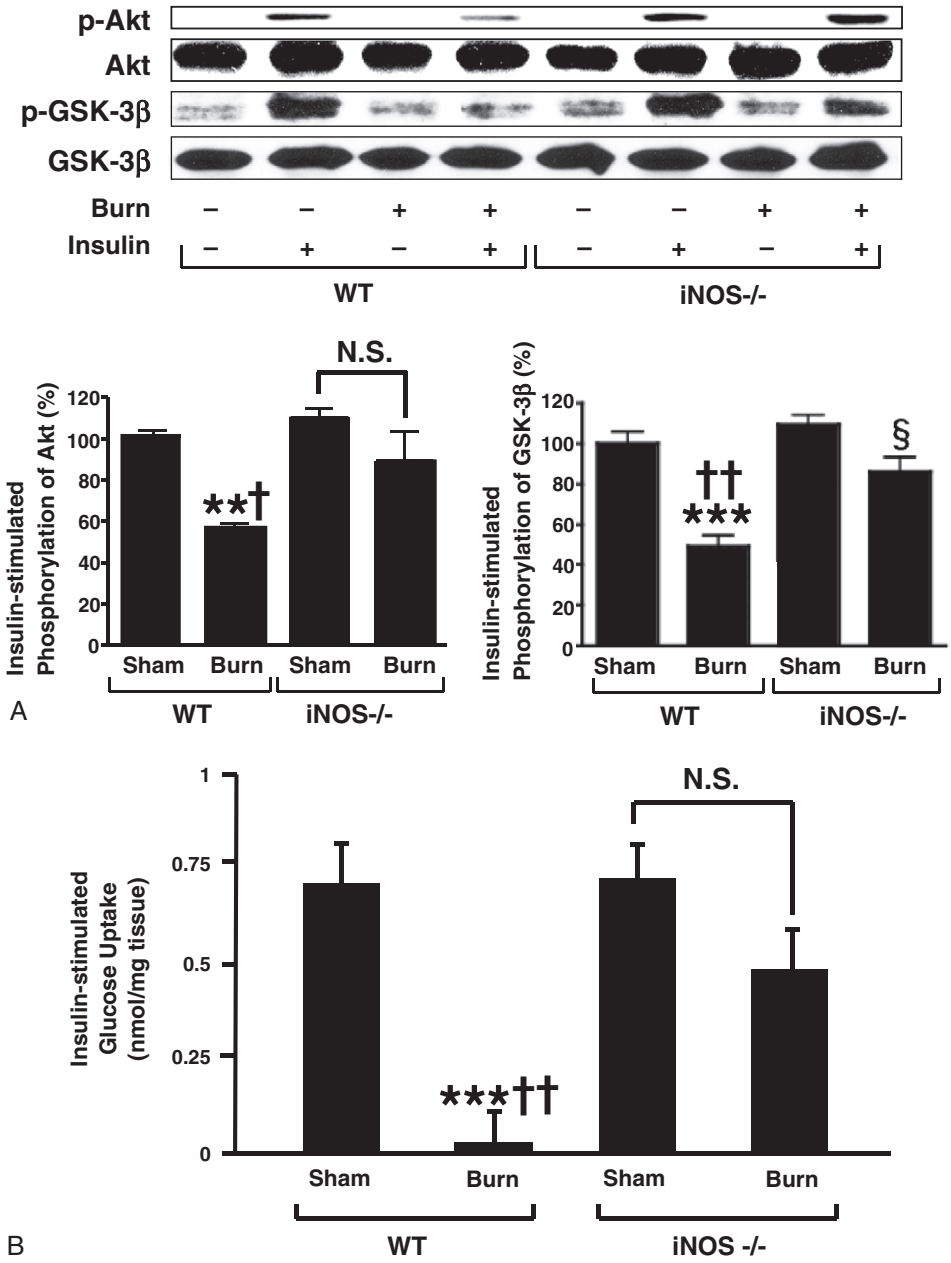

Fig. 4 – Burn injury–induced decreases in insulin-stimulated Akt phosphorylation and glucose uptake in skeletal muscle were reversed by iNOS deficiency. A, At 3 days after burn injury or sham burn, insulin (1 U/kg BW) or saline was injected; and 5 minutes thereafter, skeletal muscle was taken. Insulin-stimulated Akt phosphorylation was significantly attenuated by burn injury in wild-type (WT) mice as compared with sham animals. Inducible nitric oxide synthase deficiency (–/–) almost completely reversed decreased insulin-stimulated Akt phosphorylation in burned mice. Similarly, insulin-stimulated GSK-3ß phosphorylation was significantly decreased by burn injury in wild-type mice. Burn injury–induced decreased GSK-3ß phosphorylation was ameliorated by iNOS deficiency. Neither burn injury nor iNOS deficiency altered Akt or GSK-3ß protein expression. The overall interactions between genotype and burn/sham are statistically significant for phosphorylation of Akt ($P < .01$) and GSK-3ß ($P < .05$). **$P < .01$, ***$P < .001$ vs sham-burned WT; †$P < .05$, ††$P < .01$ vs burned iNOS –/–; §$P < .05$ vs sham-burned iNOS –/–. NS indicates not significant. n = 4 per group of animals with saline; n = 6 per group of animals with insulin. B, Burn injury resulted in suppression of insulin-stimulated glucose uptake by skeletal muscle ex vivo. Inducible nitric oxide synthase deficiency significantly improved insulin-stimulated glucose uptake in burned mice. The interaction between genotype and burn/sham is statistically significant ($P < .05$). ***$P < .001$ vs sham-burned WT, ††$P < .01$ vs burned iNOS–/–. NS indicates not significant. n = 4 to 6 per group.

of GSK-3β. Burn injury decreased the insulin-stimulated phosphorylation of GSK-3β in wild-type, but not iNOS knockout, mice. The protein expression of Akt and GSK-3β was not affected by burn injury or iNOS deficiency.

To further investigate the biological relevance of the amelioration of impaired insulin signaling by iNOS deficiency in burned mice, we evaluated insulin-stimulated glucose uptake in skeletal muscle ex vivo. In wild-type mice, insulin-stimulated glucose uptake was suppressed by burn injury compared with sham-burn. Gene disruption of iNOS almost completely restored insulin-stimulated glucose uptake in skeletal muscle of burned mice to the levels observed in sham animals (Fig. 4B). In contrast, iNOS deficiency did not affect insulin-stimulated glucose uptake in sham-burned animals.

3.4. *iNOS deficiency reversed whole-body insulin resistance, but did not ameliorate decreased blood glucose levels in burned mice*

Burn injury was associated with whole-body insulin resistance in wild-type mice, as judged by insulin tolerance test. Gene disruption of iNOS reverted burn injury–induced insulin resistance (Fig. 5A). Unexpectedly, blood glucose levels were lower in burned mice compared with sham animals (Fig. 5B). Inducible nitric oxide synthase deficiency did not affect blood glucose levels in either burned or sham-burned mice. Neither burn injury nor iNOS deficiency significantly altered plasma insulin concentrations in mice (Fig. 5C). We evaluated mRNA expression levels of PEPCK and G-6-Pase, gluconeogenic genes, in the liver. The PEPCK and G-6-Pase mRNA levels did not significantly differ between the groups (PEPCK mRNA [%]: WT sham: 100 ± 13 [mean ± SEM]; WT burn: 102 ± 8; KO sham: 101 ± 13; KO burn: 121 ± 9; G-6-Pase [%]: WT sham: 100 ± 16; WT burn: 85 ± 10; KO sham: 87 ± 11; KO burn: 80 ± 10).

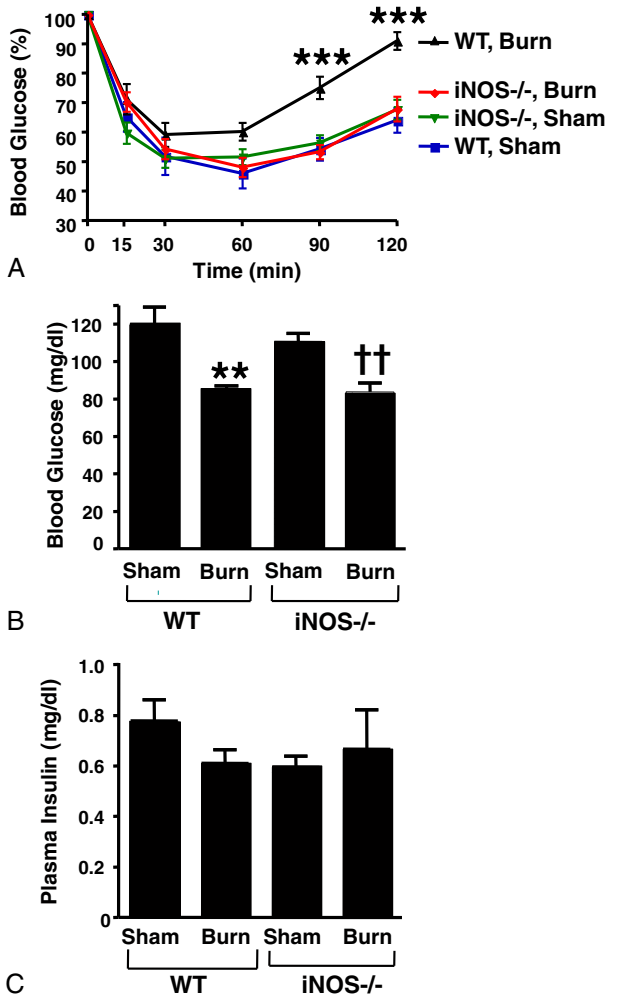

Fig. 5 – Inducible nitric oxide synthase deficiency improved whole-body insulin resistance in burned mice, but did not alter decreased blood glucose levels after burn injury. A, Insulin tolerance test revealed that hypoglycemic response to insulin injection was significantly blunted in burned wild-type (WT) compared with sham animals. Gene disruption of iNOS (–/–) restored sensitivity to insulin-stimulated decrease in blood glucose levels in burned mice. The overall interactions between genotype and burn/sham at 90 and 120 minutes after the insulin injection are statistically significant (P < .005). ***P < .001 vs sham-burned WT and sham-burned and burned iNOS–/–. n = 8 per group. B, Unexpectedly, burn injury resulted in decreased blood glucose levels in both wild-type and iNOS-deficient mice. Inducible nitric oxide synthase deficiency did not alter blood glucose levels either in sham-burned mice or in burned mice. **P < .01 vs sham-burned WT, ††P < .01 vs sham-burned iNOS–/–. n = 8 per group. C, Neither burn injury nor iNOS deficiency significantly affected plasma insulin concentrations.

4. Discussion

We found that gene disruption of iNOS significantly ameliorated impaired IRS-1–mediated insulin signaling in skeletal muscle of burned mice. Consistent with our previous studies in rats [17,32], burn injury caused decreases in insulin-stimulated tyrosine phosphorylation of IR and IRS-1 (Figs. 1, 2) and insulin-stimulated activation of PI3K (Fig. 3) and Akt (Fig. 4A) in skeletal muscle compared with sham-burn. Burn injury caused a marked induction of iNOS at 3 days after burn (Fig. 1A). Importantly, the impairment of IR/IRS-1–mediated insulin signaling in skeletal muscle paralleled iNOS induction following burn injury (Fig. 1). The insulin-stimulated tyrosine phosphorylation of IR and IRS-1, insulin-stimulated PI3K activity, insulin-stimulated Akt phosphorylation, and IRS-1 expression were significantly greater in burned iNOS–/– mice compared with burned wild-type mice (Figs. 1-4). Likewise, burn injury resulted in suppressed insulin-stimulated glucose uptake by muscle, which was reverted by iNOS deficiency (Fig. 4B). These findings clearly indicate that iNOS plays an important role in burn injury–induced insulin resistance in mouse skeletal muscle.

Mitigation of impaired insulin signaling by gene disruption of iNOS was observed at the levels of both IR and IRS-1 in skeletal muscle of burned mice. In insulin-resistant states, the impaired insulin-stimulated tyrosine phosphorylation of

IRS-1, a major component of insulin resistance, may result from decreased tyrosine phosphorylation of IRS-1 by IR kinase, reduced IRS-1 expression, or a combination of both. Both attenuated IR kinase activity, reflected by reduced insulin-stimulated tyrosine phosphorylation of IR, and decreased IRS-1 expression were associated with insulin resistance in skeletal muscle of burned mice. It should be noted, however, that when normalized to IRS-1 protein expression, the ratio of insulin-stimulated IRS-1 phosphorylation vs IRS-1 protein expression was not altered by iNOS deficiency in either sham or burned animals (Fig. 2E). These findings suggest that the improved insulin-stimulated phosphorylation of IRS-1 by iNOS deficiency may be mainly attributable to the increased IRS-1 protein expression in skeletal muscle of burned mice.

Improvement of reduced IRS-1 expression by iNOS deficiency was in agreement with our previous study that showed that iNOS and NO donor reduce IRS-1 expression in cultured skeletal muscle cells and that iNOS deficiency restored the reduced IRS-1 expression in skeletal muscle of genetically obese, diabetic (*ob/ob*) mice [28]. Taken together, these observations suggest that iNOS expressed in skeletal muscle may contribute to reduced IRS-1 protein expression following burn injury, as seen in obesity.

With respect to tyrosine phosphorylation of IR, the effects of iNOS deficiency on insulin signaling seem to differ between burn injury- and obesity-induced insulin resistance. In burned mice, gene disruption of iNOS significantly improved insulin-stimulated phosphorylation of IR as well as IRS-1 (Fig. 2). In contrast, iNOS and NO donor did not attenuate insulin-stimulated tyrosine phosphorylation of IR in cultured skeletal muscle cells [28]. Consistent with the observation in cultured cells, iNOS deficiency did not significantly alleviate the reduced insulin-stimulated tyrosine phosphorylation of IR in obese, diabetic (*ob/ob*) mice [28]. One may speculate, therefore, that the improvement in insulin-stimulated tyrosine phosphorylation of IR in burned iNOS–/– mice may not be fully attributable to locally expressed iNOS in the skeletal muscle. Our preliminary observation revealed that iNOS expression was also induced in adipose tissue and liver at 3 days after burn injury in mice (unpublished observation 2009, M. Kaneki). Hence, it is possible that iNOS induction in tissues other than skeletal muscle, including adipose tissue, might also contribute to insulin resistance in skeletal muscle, particularly attenuated insulin-stimulated tyrosine phosphorylation of IR in burned mice. Alternatively, differences in the degree of iNOS induction in skeletal muscle of burned mice vs obese, diabetic (*ob/ob*) mice might contribute to the distinct effects of iNOS on IR phosphorylation. Inducible nitric oxide synthase expression following burn injury is much greater than that observed in skeletal muscle of obese, diabetic (*ob/ob*) mice.

Insulin resistance of critical illness lasts longer [1,2,38] than the 3-day period observed in the present study. In fact, reduced IRS-1 expression was also observed at 7 days after burn injury (end of observation period of this study) (Fig. 1). The magnitude and size of the body surface burned area in this study were quite small (12%), where the inflammatory process was modest and self-limiting. A longer-lasting effect on insulin signaling would be demonstrated in a more serious injury model [18,19].

In combination with insulin insufficiency (pancreatic β-cell dysfunction), insulin resistance is a major causative factor for hyperglycemia. It is interesting to note, however, that whole-body and skeletal muscle insulin resistance was accompanied by decreased blood glucose levels in burned wild-type mice compared with sham animals. Although gene disruption of iNOS reversed insulin resistance, it did not affect blood glucose levels in burned mice. The decreased blood glucose level in burned mice contrasts with our previous findings of burn injury–induced hyperglycemia and hyperinsulinemia in rats [32]. A recent study has also shown that burn injury results in a modest increase in blood glucose levels at 7 days after burn injury in CD1 mice [39]. This apparent discrepancy in the effects of burn injury on blood glucose levels could be explained by differences in the experimental procedures, including burn injury, and differences in species and strains of rodents. It is important to note that hypoglycemia also occurs in burn patients without insulin treatment or other hypoglycemic agents [40,41], although in many cases burn injury is associated with hyperglycemia in humans. We do not presently have an explanation why the burned mice developed hypoglycemia; but because the reduced blood glucose levels were not influenced by iNOS deficiency, the hypoglycemia was probably unrelated to the burn-induced increase in iNOS expression. Further studies are required to clarify the etiology of hypoglycemia in burns.

Our data clearly indicate that iNOS plays an important role in burn injury–induced skeletal muscle insulin resistance. These results suggest that iNOS may exert insulin-desensitizing effects, at least in part, by suppressing the protein expression of IRS-1 in skeletal muscle after burn injury.

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Conflict of Interest

No conflict of interest.

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