Diabetologia (1998) 41: 1523–1527

Diabetologia

Rapid communication

Insulin inhibits inducible nitric oxide synthase in skeletal muscle cells

S. Bédard, B. Marcotte, A. Marette

Department of Physiology and Lipid Research Unit, Laval University Hospital Research Center, Ste-Foy, Québec, Canada

Summary Recent studies have shown that cytokines and endotoxins impair insulin-stimulated glucose transport by activating the expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production in skeletal muscle cells. In this study, we investigated whether iNOS induction is modulated by insulin in L6 myocytes. Long term exposure of muscle cells to tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and lipopolysaccharide (LPS) greatly increased iNOS mRNA expression and NO production. Addition of insulin to the cytokine/LPS-treated muscle cells reduced (by ~ 40 %) NO production. This inhibition was similar to that observed with the synthetic glucocorticoid dexamethasone, a known inhibitor of iNOS in several cell types. The combination of insulin and dexamethasone was more effective than either agent alone in reducing NO production. Dexamethasone greatly inhibited the effect of cytokines/LPS to induce cellular iNOS mRNA expression. In strong contrast, insulin failed to reduce iNOS mRNA expression under similar conditions. These results show that insulin is a novel inhibitor of iNOS-mediated NO production in skeletal muscle cells. Furthermore, our data indicate that unlike glucocorticoids, insulin does not inhibit NO production by suppression of iNOS gene transcription. [Diabetologia (1998) 41: 1523–1527]

Keywords Insulin, nitric oxide, nitric oxide synthase, skeletal muscle, glucocorticoids, cytokines, lipopolysaccharide.

Bacterial endotoxins and inflammatory cytokines are potent inducers of inducible nitric oxide synthase (iNOS) activity and cellular expression. Such stimulation has been reported in macrophages, where nitric oxide (NO) production is primarily responsible for their antimicrobial action and also in various cell types including skeletal muscle cells [1]. Although iNOS is detected at rather low concentrations in intact skeletal muscle, its expression is greatly increased after in vivo treatment with lipopolysaccharide (LPS) [1–4] or in vitro exposure of skeletal muscle cells to cytokines or LPS or both [5–7]. We have recently shown that iNOS induction is associated with impaired insulin-stimulated glucose transport in skeletal muscle [2, 5]. Moreover, iNOS inhibition by N^G-nitro-L-arginine methyl ester (L-NAME) restored insulin action in cytokine-treated muscle cells [5]. These studies indicate that iNOS-mediated NO production reduces insulin action in skeletal muscle cells. It has been shown that iNOS induction is responsible for the impairment in muscle contractility that is observed in endotoxin shock [3, 8]. A role for iNOS has also been proposed in causing muscle injury following ischaemia-reperfusion [9].

It has been shown that iNOS expression is modulated by glucocorticoids in several cell types [1, 10–12]. Recent studies further indicated that peptidic hormones, such as glucagon, can also inhibit iNOS expression in hepatocytes [13]. Insulin was found to

Received: 3 June 1998 and in revised form: 24 July 1998

Corresponding author: Dr. A. Marette, Lipid Research Unit, Laval University Hospital Research Center, Ste-Foy, Québec, G1V 4G2, Canada

Abbreviations: NO, Nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; L-NAME, N^G-nitro-L-arginine methyl ester; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; GAPDH, glyceraldehyde phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.

have no effect on liver iNOS expression in the same study. On the other hand, the hormonal modulation of skeletal muscle iNOS is still poorly understood. The aim of this study was to determine whether insulin modulates iNOS expression in skeletal muscle cells and to compare this effect with that of the synthetic glucocorticoid dexamethasone.

Materials and methods

Cell culture and treatment. A line of L6 skeletal muscle cells (kind gift of Dr. A. Klip, Hospital for Sick Children, Toronto, Canada) clonally selected for high fusion potential was used in our study. Cells were grown and maintained in monolayer culture in alpha minimum essential medium (α-MEM) containing 2% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antimycotic solution (10000 units/ml penicillin, 10000 μg/ml streptomycin and 25 μg/ml amphotericin B) in an atmosphere of 5% CO_2 at 37°C. We plated L6 myoblasts in 10 cm dishes at 10^5 cells/ml and used these after complete differentiation to myotubes (7 days post-plating), as described previously [5]. We incubated L6 myotubes for 24 h with or without tumour necrosis factor-α (TNF-α) (10 ng/ml, 0.6 nmol/l), interferon-γ (IF-γ) (200 U/ml, 1 nmol/l) and LPS (10 μg/ml), and in the absence or presence of insulin (human Humulin R) or dexamethasone (concentrations indicated in figure legends) or both. In some experiments, the NOS inhibitor L-NAME (2 mmol/l) was also added with cytokines and LPS. The incubation medium was then removed for analysis of nitrite and cells were immediately used for RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR), as described below.

Nitrite production. The accumulation of nitrite in the incubation medium was used as an index of NO production by iNOS. Nitrite production was fully inhibited in the presence of 2 mmol/l L-NAME. Furthermore, only iNOS can produce NO in the L6 myocytes since neither control nor cytokine/LPS-treated cells express the other nitric oxide synthase isoforms eNOS and nNOS [2, 5]. Nitrite was determined spectrophotometrically using the Griess reagent as described previously [5]. Briefly, 100 μl of the Griess reagent (1.0% Sulphanilamide/ 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) was added to 100 μl samples of the incubation medium in 96-well plates. The absorption was read at 540 nm against standard curves of sodium nitrite.

RNA extraction and RT-PCR. Total cellular RNA was isolated using guanidium thiocyanate-phenol-chloroform extraction with the TRIzol Reagent (Life Technologies, Burlington, Ontario, Canada) based on a method developed previously [14]. We did cDNA synthesis and amplification exactly as described previously [5]. Sequences of the antisense and sense oligonucleotides [based on rat iNOS and glyceraldehyde phosphate dehydrogenase (GAPDH)] were as follows; iNOS: 5′-TGGAACCACTCGTACTTGGGA-3′ and 5′-CAAGAGTTTGACCAGAGGACC-3′, GAPDH: 5′-AGATCCACAACGGATACATT-3′ and 5′-TCCCTCAAGATTGTCAGCAA-3′. The expected sizes of amplification products were 653 base pairs for iNOS and 331 base pairs for GAPDH. Amplification products were run in 8% acrylamide gels, stained in ethidium bromide and fluorescence associated with DNA bands was analysed by laser scanning densitometry using a tabletop Agfa scanner (Arcus II, Etibicoke, Ontario, Canada) and quantitated with the NIH Image program (available on Internet by anonymous FTP from zippy.nimh.nih.gov.).

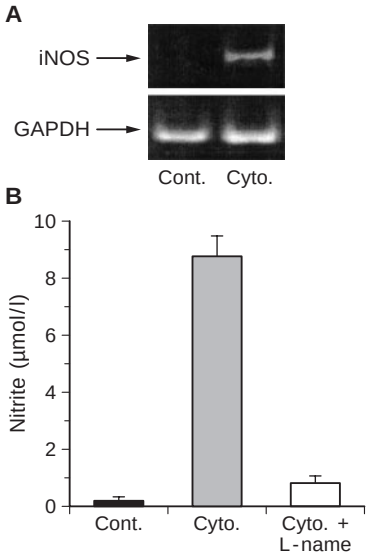

Fig. 1 A, B. Effect of cytokines and LPS on iNOS mRNA expression and nitrite production in L6 myocytes. L6 cells were treated (CYTO) or not (control) with TNF-α (10 ng/ml, 0.6 nmol/l), IFN-γ (200 units/ml, 1 nmol/l), LPS (10 μg/ml) for 24 h, followed by (**A**) RNA isolation and RT-PCR analysis of iNOS mRNA expression. GAPDH mRNA expression was also measured in the same samples. The sizes of amplification products were, as expected, 653 bp for iNOS and 331 bp for GAPDH. (**B**) Measurement of nitrite production in the incubation medium. In some experiments, the NOS inhibitor L-NAME was also added to the cells (CYTO + L-NAME). Results are representative of 6 individual experiments

Statistical analysis. Values are means ± SEM. The effects of insulin or dexamethasone or both on NO production were compared by a two-way analysis of variance (ANOVA). The level of significance was $p < 0.05$.

Results

The cytokine/LPS mixture greatly induced iNOS expression as reflected both by an increase in iNOS mRNA expression (Fig. 1 a) and nitrite accumulation in the incubation medium (Fig. 1 b). These effects were specific for iNOS since the expression of the house-keeping GAPDH gene was not affected in the same cells. Furthermore, nitrite accumulation was prevented when cells were incubated with cytokines/LPS and the NOS inhibitor L-NAME confirming that iNOS activity was responsible for the production of nitrite. Other NOS isoforms are not expressed in these cells [5].

Addition of insulin to cytokine/LPS-exposed muscle cells blunted NO production (to 63 ± 8% of control) (Fig. 2). As reported previously for other cell types, dexamethasone also inhibited NO release by

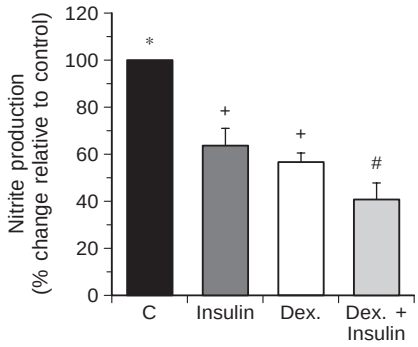

Fig. 2. Effects of insulin or dexamethasone or both on cytokines/LPS-induced NO production in L6 myocytes. L6 cells were treated with cytokines/LPS for 24 h as described in Figure 1. Cells were incubated with cytokines/LPS in the absence (C) or presence of insulin (INS) 0.6 μmol/l or dexamethasone (DEX) 1 μmol/l or both before measurement of nitrite. The bars represent means ± SEM of 6 individual experiments done in triplicate. Nitrite production in cytokines/LPS-treated cells was 8.8 ± 0.7 μmol/l. * + # indicate a statistical significance between the experimental groups. Bars not sharing a common superscript are different at $p < 0.05$

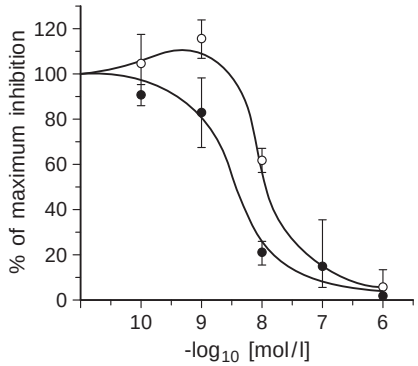

Fig. 3. Dose-response relations for the effects of insulin or dexamethasone on cytokines/LPS-induced NO production in L6 myocytes. L6 cells were treated with cytokines/LPS with or without different concentrations of insulin (●, INS) or dexamethasone (○, DEX) for 24 h before measurement of nitrite concentrations in the incubation medium. Results are expressed relative to the maximum inhibition observed at 1 μmol/l of insulin or dexamethasone. Nitrite production in cytokines/LPS-treated cells was 8.8 ± 0.7 μmol/l. The points represent the means ± SD of 2 individual experiments done in triplicate

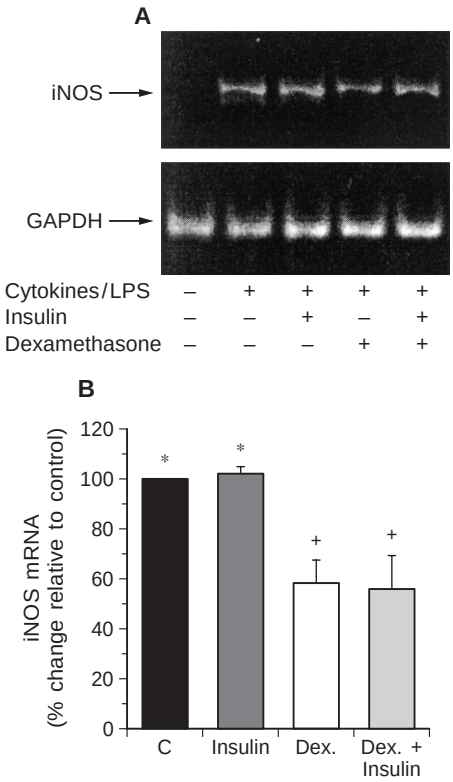

Fig. 4 A, B. Effect of insulin or dexamethasone or both on cytokines/LPS-induced iNOS expression in L6 myocytes. L6 cells were treated with or without cytokines/LPS, insulin 0.6 μmol/l or dexamethasone 1 μmol/l or both for 24 h before RNA isolation and RT-PCR analysis of iNOS and GAPDH mRNAs as in Figure 1. (**A**) Representative gels showing iNOS and GAPDH amplified products. (**B**) Means ± SEM of 6 individual experiments. *+ indicates a statistical significance between the experimental groups. Bars not sharing a common superscript are different at $p < 0.05$

myocytes (to 56 ± 3 % of control). The combination of insulin and dexamethasone treatments was more effective in blocking NO production (to 40 ± 7 % of control) than either treatment alone. Insulin was more potent than glucocorticoids at inhibiting NO production (Fig. 3). Insulin's ability to reduce NO production was already detectable at 0.1 nmol/l and almost reached maximum at 10 nmol/l, whereas dexamethasone inhibition of cytokine-induced NO release was barely detectable at 10 nmol/l and clearly observed at doses of more than 100 nmol/l.

Insulin failed to affect iNOS mRNA induction in cytokine/LPS-treated cells. In strong contrast, dexamethasone reduced iNOS gene expression in the same cells (to 58 ± 8 % of control). The effect of both agents were identical to that of dexamethasone alone. Expression of GAPDH mRNA was not affected in any of those experimental conditions (Fig. 4).

Discussion

These results show for the first time that insulin can directly modulate iNOS activity in myocytes. Such direct modulation of iNOS activity by insulin has not been reported for other cell types. In a recent study, it was found that glucagon inhibits cytokine-induced NO production in hepatocytes but that insulin is without effect [13]. Thus, our results suggest that iNOS modulation by insulin is specific to muscle cells and not a characteristic of all insulin-sensitive cells. Insulin has been shown to directly increase endothelium NO production in isolated arterioles and in human

umbilical vein endothelial cells in culture, suggesting that the hormone activates the endothelial type NOS isoform (eNOS) in these cells [15, 16]. Thus, insulin regulation of NOS activity appears to be isoform-specific.

The effect of insulin on NO production was similar to that of dexamethasone but the mechanisms implicated are clearly different. Both treatments reduced iNOS-mediated NO production by about a half but only dexamethasone caused a decrease in iNOS mRNA and the blunting effect of dexamethasone on myocyte NO production could be entirely explained by the suppression of iNOS expression. The partial additivity of insulin and dexamethasone effects on NO production is also consistent with the proposition that two different mechanisms are implicated.

Insulin's ability to inhibit iNOS activity in skeletal muscle could be relevant to the pathogenesis of diabetes. We have reported recently that cytokines induce iNOS expression and that LPS synergistically enhances this effect in L6 myocytes [5]. Induction of iNOS in cytokine-treated muscle cells was associated with a reduced ability of insulin to increase glucose transport in these cells but basal glucose transport activity was augmented in the same condition. These effects could be totally reversed by NOS blockade with L-NAME, implicating that iNOS-mediated NO production was responsible for the alterations in glucose transport activity. Furthermore, LPS challenge in rats also induced muscle iNOS expression [1–4, 6, 17] and NO production by isolated muscles [2] which were associated with an impaired insulin-mediated glucose uptake in the same muscles [2]. Increasing NO to the concentrations seen in cytokine/LPS-treated cells by exogenous addition of NO donor drugs to isolated rat muscles or cultured L6 myocytes also blunted insulin action on glucose transport and amino acid uptake [2]. On the other hand, the present data show that insulin is able to inhibit the action of cytokines to increase iNOS-mediated NO production and is thus a potent modulator of iNOS in skeletal muscle cells. More studies are needed to confirm the effect of insulin on iNOS activity in skeletal muscle tissue in vivo. Nevertheless, the present data suggest that under normal physiological conditions, muscle iNOS activity is inhibited by circulating insulin. Induction of iNOS in various disease states is associated with both an increase in inflammatory mediators and a reduction in insulin concentrations or action. Thus, the lack of insulin in Type I (insulin-dependent) diabetes mellitus (autoimmune or induced by streptozotocin) is associated with increased macrophage iNOS activity [18]. Moreover, we have observed that skeletal muscle from streptozotocin-induced diabetic rats increased iNOS (Ca^{2+}-independent) activity (unpublished data). Increased muscle iNOS expression in sepsis is also associated with a relative insulin resistance (i. e. lack of insulin action) at the cellular level. Thus, iNOS activity in the above pathological states may or could be induced by the combination of increased concentrations of inflammatory cytokines and diminished insulin concentrations or action. It is noteable that obesity-linked diabetes is also associated with increased concentrations of TNF-α and impaired insulin action in both muscle and adipose cells [19, 20]. It will be important to test whether muscle and adipose cells from obese subjects have increased iNOS activity, and if insulin's ability to inhibit the enzyme is impaired in these cells.

Acknowledgements. This work was supported by grants from the Medical Research Council of Canada and the Canadian Diabetes Association. S. Bédard was supported by a FCAR-FRSQ studentship. A. Marette holds a scholarship from the Canadian Medical Research Council.

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