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Diminished production of nitric oxide synthase cofactor tetrahydrobiopterin by rosiglitazone in adipocytes

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Abstract

Increased nitric oxide (NO) synthesis has been proposed to participate in the generation of insulin resistance in adipose and muscle tissues. Therefore, we examined the potential rate-limiting role of tetrahydrobiopterin (BH4) in cytokine-induced NO synthesis, and the effect of peroxisome proliferator activated receptor-γ (PPARγ) activation using the insulin-sensitizer rosiglitazone on cytokine-induced BH4 synthesis in 3T3-L1 adipocytes. Our data indicate that modulated availability of the mandatory nitric oxide synthase (NOS) cofactor BH4 affected cytokine-induced NO generation. Semiquantitative linear range reverse transcription polymerase chain reaction (RT-PCR) analysis demonstrated that rosiglitazone not only reduced inducible nitric oxide synthase (iNOS) mRNA transcription, but also guanosine triphosphate cyclohydrolase (GTPCH), the rate-limiting and controlling step of BH4 synthesis. Accordingly, intracellular BH4 concentration was reduced by 45% following rosiglitazone treatment. Furthermore, we observed a transient inhibitory effect of natural PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PJ2) on cytokine-mediated iNOS and GTPCH induction. Thus, the inhibition of cytokine-induced NO synthesis by rosiglitazone is at least in part attributable to reduced availability of BH4, the synthesis of which might represent a potential new target in the treatment of type 2 diabetes and insulin resistance.

Keywords: Nitric oxide; Tetrahydrobiopterin; Thiazolidinediones; Rosiglitazone; Insulin resistance; Adipocytes

1. Introduction

NO is a messenger for a wide variety of physiological functions [1]. In recent years human [2] and rodent [3,4] adipose tissue has emerged as a potential site of nitric oxide production. NO derived nitrite and nitrate concentrations correlate with body fat composition in humans [5]. Conflicting results were reported regarding NO as a modulator of insulin-mediated glucose uptake [6,7] and antilipolysis in adipocytes [8,9]. NOS2$^{-/-}$ knockout mice unable to express iNOS are protected from obesity-induced insulin-resistance [10]. Furthermore, treatment with thiazolidinediones inhibits cytokine-mediated induction of iNOS expression in macrophages [11] and in 3T3-L1 adipocytes [12]. Thiazolidinediones are a new class of antidiabetic drugs ameliorating insulin sensitivity *in vivo* [13] and preventing cytokine- or dexamethasone-induced insulin resistance *in vitro* [14,15]. Their mode of action includes the activation of PPARγ, a signal molecule expressed at particularly high levels in adipocytes [16].

BH4 is a mandatory cofactor of all NOS isoforms [17]. It stabilizes active NOS dimers [18] and has a role in catalytic electron flow towards substrate L-arginine [19]. BH4 appears to have a pivotal role in NO signaling since its availability is rate-limiting in nitric oxide synthesis in many cells [20]. BH4 depletion is crucial in the control of both NO and superoxide generation by NOS isoforms, and consequently on the formation of cell toxic peroxynitrite [21]. Finally, BH4 was suggested to modulate the stability of iNOS mRNA [22,23]. The level of BH4 synthesis is mainly controlled by the activity of GTPCH, the rate-limiting enzyme of the *de novo* BH4 biosynthetic pathway [20].

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Abbreviations: BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; GTPCH, guanosine triphosphate cyclohydrolase; iNOS, inducible nitric oxide synthase; IFNγ, interferon-γ; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthases; PPARγ, peroxisome proliferator activated receptor-γ; TNFα, tumor-necrosis factor-α; 15d-PJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; RT-PCR, reverse transcription polymerase chain reaction.

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The present studies were performed to determine the role of BH4 synthesis in NO metabolism in adipocytes and to define a possible role of PPARγ activation in this process by incubating the cells with rosiglitazone, a thiazolidinedione used in the treatment of insulin resistance and type 2 diabetes.

2. Materials and methods

2.1. Materials

Mouse specific cytokines were purchased from Pepro-Tech EC Ltd., E. coli 026:B6 Lipopolysacharide (LPS), dicumarol (3,3′-methylene-bis(4-hydroxycoumarin)) and chemicals were from Sigma, rosiglitazone was kindly provided by GlaxoSmithKline. Sepiapterin was from Schircks Laboratories and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 was from Alexis Biochemicals.

2.2. Cell cultures

The commercial preadipocyte cell line 3T3-L1 (ATCC) was grown in 100 mm culture dishes at 37° in DMEM (Sigma) containing 3.7 g/L $NaHCO_3$ giving a pH of 7.4 when incubated with 5% CO_2. Two days postconfluence differentiation to mature adipocytes was initiated as described previously [24]. Cells were then stimulated with mouse IFNγ (100 U/mL), mouse TNFα (10 ng/mL), mouse interleukin-1 (100 U/mL), mouse interleukin-6 (6000 U/mL) and LPS (1 μg/mL). Stimulated cells were supplemented with rosiglitazone (1 μM), dicumarol (10 μM), 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (5 μM), or sepiapterin (10 μM) as indicated.

2.3. mRNA analysis

Total cellular RNA was isolated using *RNaqeous* kit from Ambion. RNA extracts were visualized by ethidium bromide on 1.5% agarose gels and quantified by OD measurements. Aliquots containing 1 μg RNA were reverse transcribed using *Omniscript* kit (Qiagen) and oligo(dT) primers (Promega). Negative controls without MMLV reverse transcriptase were run to exclude possible genomic DNA contaminations. cDNA aliquots were subjected to polymerase chain reaction using the following primers: GTPCH, 5′-CCAAGGGATACCAGGAGA-3′ (sense) and 5′-CTCGCATTACCATGCACA-3′ (antisense) yielding a 330 bp product located at base pairs 396–726 (Genbank L09737), iNOS, 5′-GACCTGAAAGAGGAAAAGGA-3′ (sense) and 5′-CAAAGATGAGCATCATCCAGA-3′ (antisense) yielding a 479 bp product located at base pairs 232–711 (Genbank NM_010927), eNOS, 5′-CCTGGTGTTTCCAAGGGTGA-3′ (sense) and 5′-GTGGTTACAGATGTAGGTGA-3′ (antisense) yielding a 333 bp product located at base pairs 309–642 (Genbank U53142) and β-actin 5′-GTATCCTGACCCTGAAGTA-3′ (sense) and 5′-AGCACTGTGTTGGCATAGA-3′ (antisense) yielding 709 bp product located base pairs 268–976 (Genbank X03672). Number of cycles was optimized at approximately five cycles above detection limit for each primer pair in order to stop the reaction in the linear range. Amplification products were visualized by ethidium bromide on 1.5% agarose gels and Bio-Rad Gel Doc 1000 system. Serial dilutions of cDNA ranging from 1:2 to 1:16 were subjected to PCR analysis in order to insure that the amplified bands represent the levels of GTPCH, iNOS, eNOS and β-actin. Presented gel pictures were processed with Scion image 4.02 software (Scion corporation). The respective origins of PCR products were confirmed by sequencing.

2.4. Pterin analysis

Cells were treated as indicated, washed in phosphate buffered saline and scraped in 200 μL 0.1 M HCl. After three cycles of freeze/thaw and centrifugation, supernatant was oxidized with MnO_2 and deproteinized by filtration through Ultrafree-MC 10'000 NMWL filters (Millipore). Samples of 200 μL were supplemented with 15 μL 1 M HCl and pterins were analyzed by high pressure liquid chromatography as described previously [25]. Tetrahydrobiopterin was measured as total biopterin (sum of biopterin, dihydrobiopterin, and tetrahydrobiopterin).

2.5. Nitrites analysis

Nitric oxide-derived nitrites were measured in cell culture medium using Griess reagent as described previously [26].

2.6. Statistical analysis

Results are presented as means ± SEM. Groups of experiments were compared statistically using Student's *t*-test.

3. Results and discussion

In the basal state no synthesis of NO (Fig. 1) was observed in 3T3-L1 cells and BH4 concentration in cell lysates was low at approximately 0.7 nM (Fig. 2). Accordingly, iNOS mRNA was undetectable (Fig. 3) and trace amounts of GTPCH were identified only after 35 cycles of PCR (not shown). Following inflammatory stimulation with a "cytokine mix" consisting of 10 ng/mL TNFα, 100 U/mL IFNγ and 100 U/mL LPS, both iNOS and GTPCH mRNA were strongly induced (Fig. 3) leading to NO (Fig. 1) and BH4 synthesis (Fig. 2), respectively. In the presence of IFNγ and LPS both NO and BH4 generations were dose-responsive on TNFα in a range of 1–50 ng/mL (not shown). The exposure of adipocytes to combined IFNγ, LPS, IL-1 and IL-6 was without effect (Figs. 1–3). The supplementation of

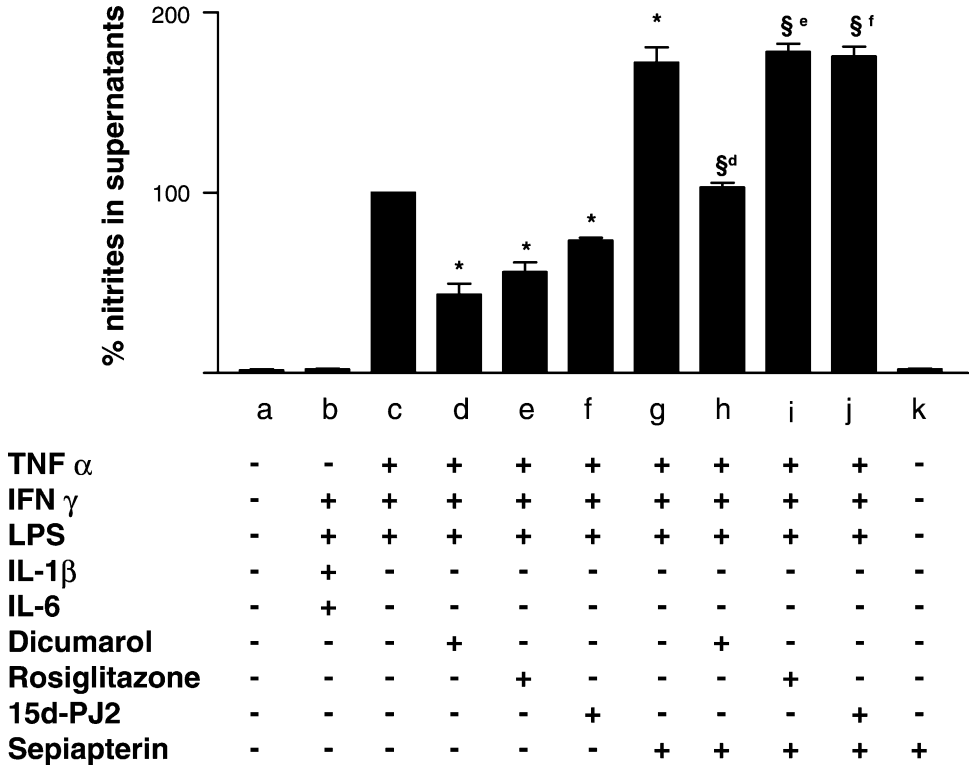

Fig. 1. Effects of modulated BH4 availability, rosiglitazone and 15d-PJ2 on cytokine-induced NO synthesis. 3T3-L1 adipocytes were treated as indicated for 24 hr. The following concentrations were used: IFNγ (100 U/mL), TNFα (10 ng/mL), IL-1 (100 U/mL), IL-6 (6000 U/mL), LPS (1 μg/mL), sepiapterin (10 μM), dicumarol (10 μM), rosiglitazone (1 μM) and 15d-PJ2 (5 μM). Accumulation of NO-derived nitrites was measured in supernatants using Griess reagent. Data from three independent experiments were normalized and presented as means ± SEM. (∗) indicates $P < 0.001$ compared to lane c. §^{d}, §^{e} and §^{f}, indicate $P < 0.001$ compared to lanes d, e and f, respectively.

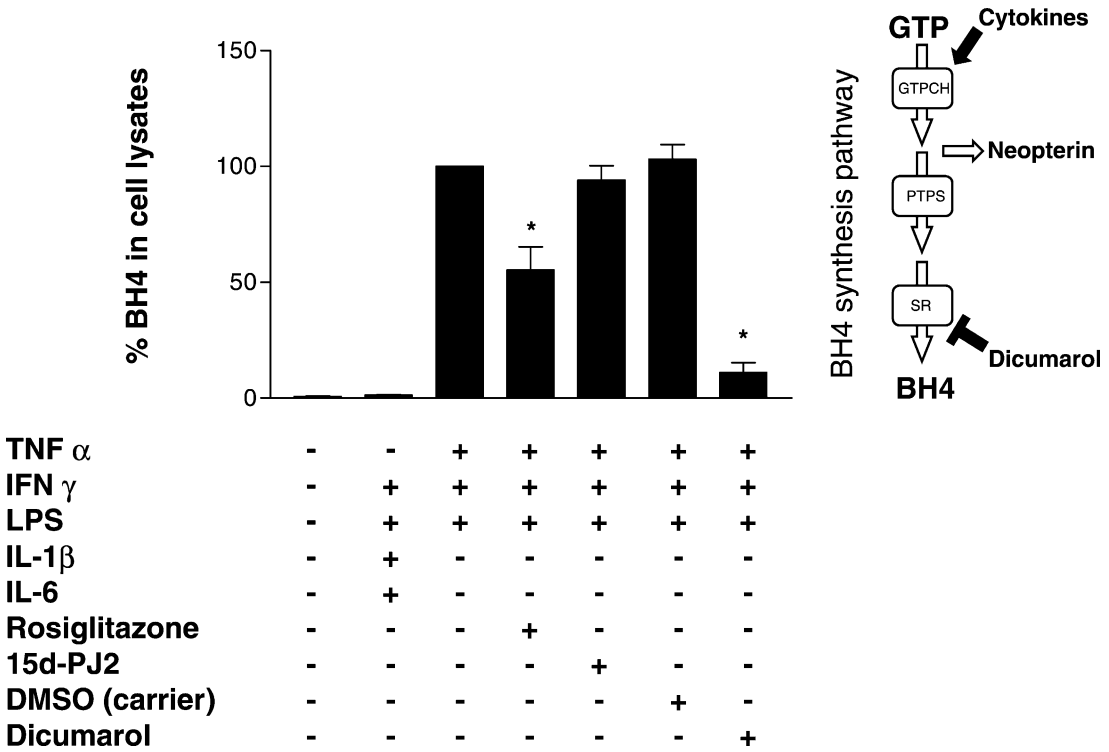

Fig. 2. Effects of rosiglitazone and 15d-PJ2 on cytokine-induced BH4 synthesis. 3T3-L1 cells were treated as indicated for 24 hr. The following concentrations were used: IFNγ (100 U/mL), TNFα (10 ng/mL), IL-1 (100 U/mL), IL-6 (6000 U/mL), LPS (1 μg/mL), rosiglitazone (1 μM) and DMSO (0.01%). Cells were lysed by freeze/thawing and BH4 content was measured in deproteinized lysates by high pressure liquid chromatography. Data from three independent experiments were normalized and presented as means ± SEM. (∗) indicates $P < 0.001$ compared to cells treated with IFNγ/TNFα/LPS.

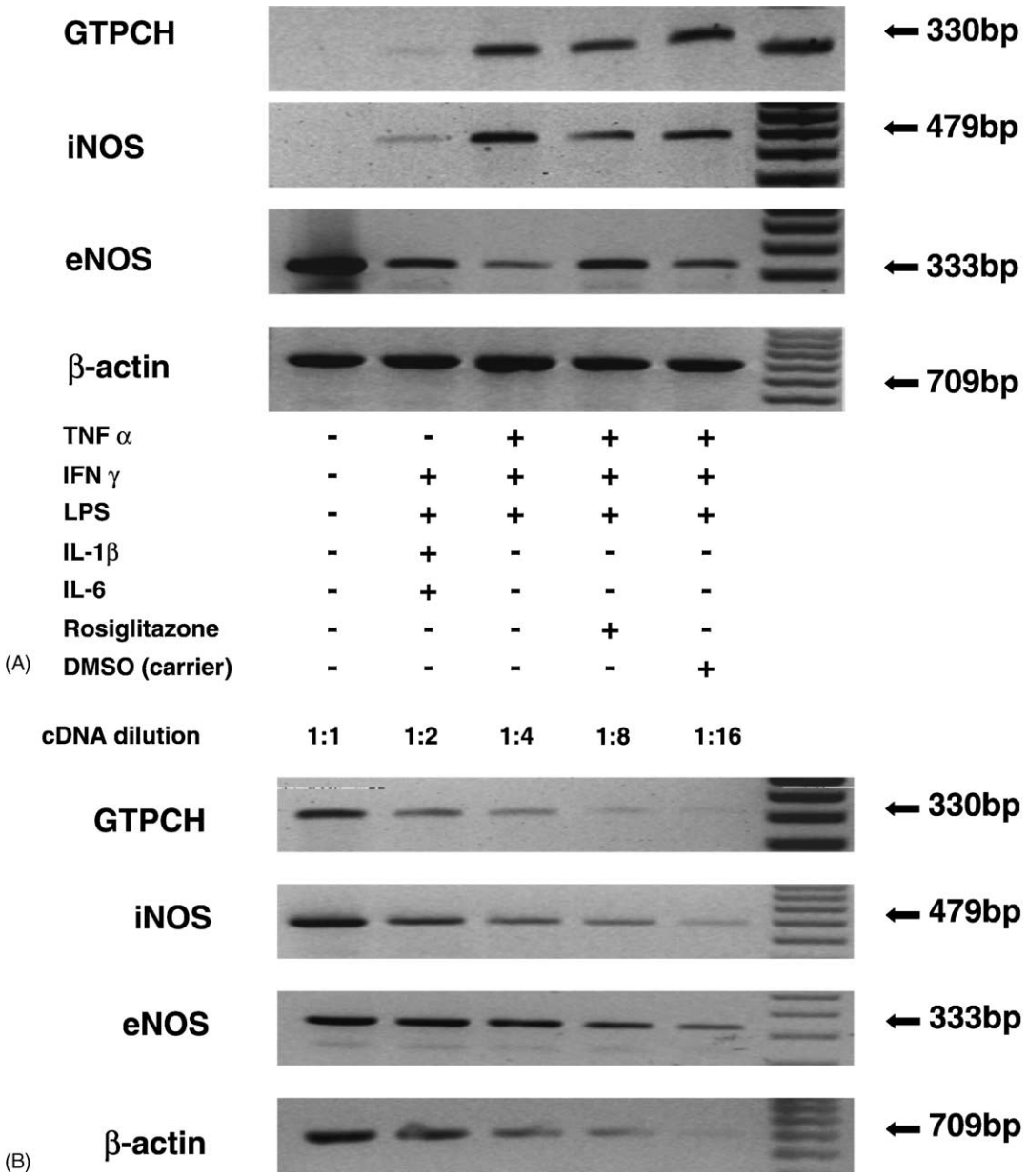

Fig. 3. Effects of cytokines and rosiglitazone on GTPCH, iNOS and eNOS mRNA expression. (A) 3T3-L1 cells were treated as indicated for 24 hr. The following concentrations were used: IFNγ (100 U/mL), TNFα (10 ng/mL), IL-1 (100 U/mL), IL-6 (6000 U/mL), LPS (1 μg/mL) and rosiglitazone (1 μM) and DMSO (0.01%). RNA was extracted from cells and subjected to linear phase RT-PCR analysis. Respective cycle numbers were as follows: GTPCH (26), iNOS (28), eNOS (35) and β-actin (24). PCR products were visualized by ethidium bromide on 1.5% agarose gels. The results presented are those of one representative out of three identical experiments. (B) cDNA was diluted as indicated and subjected to PCR analysis in order to demonstrate that the experimental conditions allowed quantitative comparison.

additional exogenous cofactor in the form of 10 μM sepiapterin, which is converted intracellularly to BH4, doubled cytokine-induced NO production (Fig. 1).

Inhibition of endogenous BH4 synthesis by 10 μM dicumarol reduced NO production by 56% (Fig. 1). The inhibitory effect of dicumarol was completely reversed by the addition of sepiapterin (Fig. 1) and large amounts of neopterin, a side product of BH4 synthesis (Fig. 2), accumulated in lysates of dicumarol treated cells (not shown). These experiments exclude nonspecific or toxic effects of the potent sepiapterin reductase inhibitor, dicumarol. Thus, BH4 appears to act as a rate-limiting cofactor on NO synthesis in the present adipocyte model, which is in accordance with other cells [20].

Cotreatment of cytokine-stimulated cells with 1 μM rosiglitazone inhibited not only NO (Fig. 1) production but also the synthesis of BH4 by 45% (Fig. 2). The down-regulating effect of the thiazoleidinedione on NO synthesis could be reversed by addition of exogenous cofactor sepiapterin (Fig. 1), suggesting that inhibition of BH4 synthesis is a critical target of the inhibitory effect of rosiglitazone on NO production. Accordingly, semiquantitative linear range

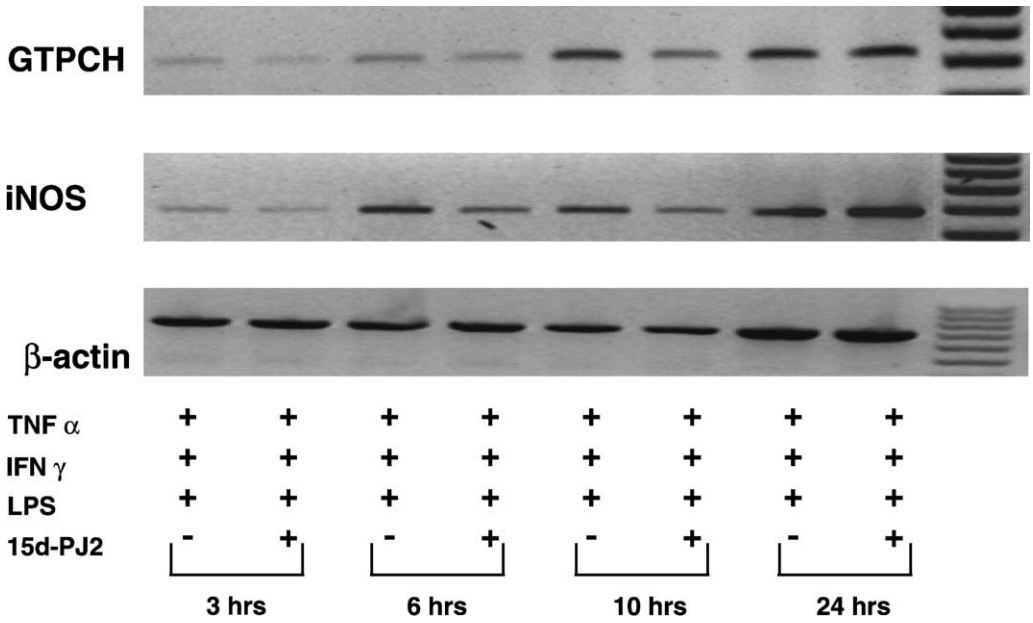

Fig. 4. Effects of 15d-PJ2 on cytokine-induced GTPCH and iNOS mRNA expression. 3T3-L1 adipocytes were treated as indicated for 3, 6, 10 and 24 hr. The following concentrations were used: IFNγ (100 U/mL), TNFα (10 ng/mL), LPS (1 μg/mL) and 15d-PJ2 (5 μM). RNA was extracted from cells and subjected to linear phase RT-PCR analysis. Respective cycle numbers were as follows: GTPCH (26), iNOS (28), β-actin (24). PCR products were visualized by ethidium bromide on 1.5% agarose gels. The results presented are those of one representative out of two identical experiments.

RT-PCR analysis demonstrated that the steady-state mRNA abundance of both iNOS and GTPCH were affected by rosiglitazone (Fig. 3).

Similar inhibitory effects on cytokine stimulated NO synthesis could be observed using the endogenous PPARγ ligand 15d-PJ2 (Fig. 1), indicating that the effects of rosiglitazone are mediated by specific participation of PPARγ. In contrast to rosiglitazone 15d-PJ2 failed to significantly suppress BH4 accumulation or GTPCH mRNA abundance after 24 hr (Figs. 2 and 4). However, the considerably different chemical stabilities of the two compounds suggest that the inhibitory effect of the rapidly degrading prostaglandin might be short lived. RT-PCR analysis following incubation periods of 3, 6 and 10 hr revealed lower cytokine-induced transcription of GTPCH and iNOS mRNAs in the presence of 5 μM 15d-PJ2 (Fig. 4), indicating a significant but transient inhibitory effect of 15d-PJ2.

eNOS mRNA was detected in untreated 3T3-L1 adipocytes (Fig. 3) and low eNOS activity was measured as accumulated nitrites in supernatants after 48 and 72 hr incubation periods (not shown), which is in accordance with other reports [7]. Following cytokine treatment we observed pronounced downregulation of eNOS transcript, which is in agreement with findings obtained in endothelial cells [27,28]. Conversely, cotreatment with rosiglitazone partially protected eNOS mRNA from downregulation (Fig. 3). Experiments using NO donors and NOS inhibitors in whole rats, muscle and adipose tissues, suggested eNOS malfunctions to cause insulin resistance in an endothelium-dependent manner [7]. Another report, however, performed on eNOS knockout mice, suggested that eNOS malfunctions occurring in insulin sensitive tissues are relevant to the generation of insulin resistance [29]. Although the eNOS-related observation described herein is limited to the transcriptional level, the present data support the hypothesis that low concentrations of eNOS-derived NO in insulin sensitive tissues may play a role in insulin signaling. In this context it is interesting to note that Elizalde *et al*. [2] reported enhanced expression of eNOS protein in adipocytes of an undefined group of obese subjects. This observation is in apparent contradiction to the fact that cytokine levels in adipose tissue of obese subjects are generally enhanced [30,31] suggesting adipose tissue-specific regulation of eNOS in humans.

iNOS-derived NO overproduction has been proposed as a possible mediator of insulin resistance in adipose and muscle tissue [5,6,8–10]. It is therefore tempting to speculate that downregulation of iNOS activity by rosiglitazone contributes to reversal of cytokine-induced insulin resistance. Here, we demonstrate that rosiglitazone not only inhibits cytokine-induced iNOS expression but also GTPCH. Thus, lower availability of the rate-limiting cofactor BH4 is partially responsible for rosiglitazone-mediated downregulation of iNOS activity. Further studies are required to confirm the possible effect of BH4 synthesis inhibition on insulin sensitivity.

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