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Induction of Adiponectin in Skeletal Muscle by Inflammatory Cytokines: *in Vivo* and *in Vitro* Studies

AURÉLIE M. DELAIGLE, JEAN-CHRISTOPHE JONAS, ISABELLE B. BAUCHE, OLIVIER CORNU, AND SONIA M. BRICHARD

Endocrinology and Metabolism Unit (A.M.D., J.-C.J., I.B.B., S.M.B.) and Orthopaedic Research Laboratory (O.C.), University of Louvain, Faculty of Medicine, 1200 Brussels, Belgium

Adiponectin (ApN) is an adipocytokine that plays a fundamental role in energy homeostasis and counteracting inflammation. We examined whether ApN could be induced in a nonadipose tissue, the skeletal muscle, *in vivo*, and in cultured myotubes in response to lipopolysaccharides or proinflammatory cytokines. We next explored the underlying mechanisms. *In vivo*, injection of lipopolysaccharides to mice caused, after 24 h, an approximately 10-fold rise in ApN mRNA abundance and a concomitant 70% increase in ApN levels in tibialis anterior muscle. This ApN induction was reproduced in C_2C_{12} myotubes cultured for 48 h with a proinflammatory cytokine combination, interferon-γ + TNFα. This effect occurred in a time- and dose-dependent manner. Several pieces of evidence suggest that nitric oxide (NO) mediates this upregulation by cytokines in myotubes or muscle. First, ApN was induced *in vitro* exclusively in the experimental conditions that stimulated NO production. Second, inducible NO synthase mRNA induction or NO production clearly preceded ApN mRNA induction. Third, preventing NO production by inhibitors of the NO synthases, nitro-L-arginine methyl ester or N^G-methyl-L-arginine, suppressed the inductive effect of the cytokines *in vitro* and *in vivo*. Finally, ApN mRNA induction by cytokines was reproduced in cultured human myotubes. In conclusion, our data provide evidence that adiponectin is up-regulated *in vivo* and *in vitro* in human and rodent myotubes in response to inflammatory stimuli. The underlying mechanisms seem to involve a NO-dependent pathway. This overexpression may be viewed as a local antiinflammatory protection and a way to deliver extra energy supplies during inflammation. (*Endocrinology* 145: 5589–5597, 2004)

ADIPOSE TISSUE SECRETES a large number of physiologically active peptides that often share structural properties with cytokines and are therefore referred to collectively as adipocytokines. Among these, adiponectin (ApN) plays a fundamental role in energy homeostasis and inflammation (1). This hormone is composed of an N-terminal collagenous domain and a C-terminal globular domain. The latter fragment, generated by proteolysis, may exert some biological effects by itself and often proves to be far more potent than full-length adiponectin on muscle (2, 3). This may be explained by the relative abundance of the two types of adiponectin receptor in this tissue. AdipoR1, which is a high-affinity receptor for globular ApN, is most abundantly expressed in skeletal muscle, whereas AdipoR2, which serves as a moderate-affinity receptor for both forms of ApN, is predominant in liver (4).

ApN exerts insulin-sensitizing properties on liver and muscle *in vivo* and *in vitro*. It enhances insulin-dependent suppression of hepatic gluconeogenesis, thereby lowering plasma glucose concentrations (5, 6). It also increases glucose uptake by C_2C_{12} myocytes or isolated mouse muscles (3, 7). ApN alters lipid metabolism as well by increasing fatty-acid oxidation in several tissues including liver and muscle, thereby accelerating the clearance of plasma free fatty acids (2, 3, 7, 8). Most of these effects are mediated by stimulation of AMP kinase and peroxisomal proliferator-activated receptor (PPAR)-α ligand activities (4, 9). Eventually, ApN exhibits antiatherogenic and antiinflammatory effects. It inhibits endothelial inflammatory response by abrogating TNFα-induced expression of adhesion molecules [through cross-talks between protein kinase A and nuclear factor-κB signaling pathways] (10, 11) and cellular superoxide generation (12). It also suppresses mature macrophage functions (phagocytic activity and TNFα production) (13). Further support for the metabolic and antiatherogenic effects of ApN comes from clinical and genetic studies. Thus, plasma ApN levels are decreased in human subjects with obesity (14), type 2 diabetes (15), or cardiovascular disease (10). Recent genome-wide scans have mapped a susceptibility locus for type 2 diabetes and metabolic syndrome to chromosome 3q27, in which the ApN gene is located (16).

Due to the potential beneficial effects of ApN, the regulation of its gene is currently being investigated. To date, this regulation has been nearly exclusively studied in the adipocyte, unique site of ApN production under normal conditions (17). However, in one study (18), it has been reported that the adiponectin gene could be induced in human myotubes exposed to an adiponectin-containing human embryonic kidney 293 cell culture supernatant.

The aim of the present work was first to examine whether adiponectin could be induced in muscle *in vivo* and cultured

Abbreviations: ApN, Adiponectin; Ct, cycle threshold; Cyclo, cyclophilin; FCS, fetal calf serum; HO-1, heme oxygenase-1; HPRT, hypoxanthine guanine phosphoribosyl transferase; IFN, interferon; iNOS, inducible NO synthase; L-NAME, nitro-L-arginine methyl ester; L-NMMA, N^G-methyl-L-arginine; LPS, lipopolysaccharides, NAC, *N*-acetylcysteine; NO, nitric oxide; NO_2^-, nitrite; NO_x, nitrate plus nitrite; PPAR, peroxisome proliferator-activated receptor; Ro, rosiglitazone; RTQ-PCR, real-time quantitative PCR.

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myotubes in response to lipopolysaccharides (LPS) or proinflammatory cytokines. We next explored the underlying mechanisms.

Materials and Methods

Animals

Male FVB or NMRI mice (27.4 g ± 0.3 g; 10 wk old; Charles River Laboratories, Brussels, Belgium) were housed at a constant temperature (22 C) with a fixed 12-h light, 12-h dark cycle. The animals received *ad libitum* a common laboratory chow (A04; UAR, Villemoisson-sur-Orge, France) until the time of the experiment.

LPS of *Escherichia coli* (serotype 0127:B8; Sigma-Aldrich, Bornem, Belgium) was dissolved in saline at a concentration of 0.1 mg/ml. At 0700 h, mice were injected ip with LPS (25 μg/animal). This dose is similar to that used by other investigators (19). In some experiments, inhibitors of the nitric oxide (NO) synthases [nitro-L-arginine methyl ester (L-NAME) or N^G-methyl-L-arginine (L-NMMA), both from Sigma-Aldrich] were dissolved in saline and injected ip at a dose of 10 mg/kg (L-NAME) or 20 mg/kg (L-NMMA) 30 min before LPS. Control mice received equivalent volumes of saline. Because LPS induces anorexia, both groups of mice were fasted throughout the experiments.

Additionally, fasted mice were injected ip with two cytokines administered simultaneously [recombinant murine interferon (IFN)γ (Peprotech, London, UK; 100 ng/animal) and TNFα (R&D Systems, Abingdon, UK; 250 ng/animal)]. The cytokines were previously dissolved in saline, each at 1 μg/ml. Control mice received the vehicle only.

Animals were killed by decapitation at various times after the injections. Blood samples were saved and tibialis anterior muscles were dissected, weighted, frozen in liquid nitrogen, and stored at −80 C until RNA extraction.

Muscles used for determination of ApN protein content were first flushed with saline to avoid tissue contamination by circulating ApN. To this end, mice were anesthetized [ketamine (Ketalar, Parke Davis division of Warner-Lambert, Zaventem, Belgium), 50 mg/kg, ip; medetomidin (Domitor, Pfizer, Brussels, Belgium), 1 mg/kg, ip], and a dilute heparin-saline solution (500 IU/ml) was injected in a backward fashion (*i.e.* into hind limbs) through a 26G catheter inserted into the posterior vena cava, the aorta being transected. Tibialis anterior muscles were then dissected and stored, as described above.

The University Animal Care Committee has approved all procedures.

Cell culture

Myoblasts from the muscle-derived C_2C_{12} cell line were obtained from American Type Culture Collection (Manassas, VA). The seeding density used throughout the experiments was 8×10^4 cells/plate of 35 mm diameter. Undifferentiated cells were grown at 37 C in the presence of 5% CO_2 in DMEM supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin, 1% nonessential amino acids, and 2% L-glutamine (all from Life Technologies, Inc., Merelbeke, Belgium). When cells reached 90–100% confluence (after 3 d), 10% heat-inactivated FCS was replaced by 2% heat-inactivated horse serum to induce myogenic differentiation. This medium will be referred to as basal medium. Muscle cells were examined for evidence of myotube formation and growth by use of an inverted IMT microscope (Olympus Optical, Hamburg, Germany). To preserve the characteristics of the C_2C_{12} cell line, the splitting of cells was done up to a maximum of seven times. The medium was changed every 48 h, and differentiation was allowed to continue for 96 h (4 d) before the experimentation period.

Primary cultures of human skeletal muscle cells were initiated from satellite cells of quadriceps samples obtained from organ donors (three men, 39 ± 9 yr). The study had the approval of the local ethical committee. Cultures were performed as described (20). Briefly, muscle biopsies were trimmed of fat and connective tissue, and then minced into 1-mm^3 fragments in a calcium- and magnesium-free saline solution. Satellite cells were isolated by trypsin digestion at 37 C. The supernatants of successive dissociations were centrifuged (200 × *g* for 5 min) and the pellet resuspended in growth medium containing Ham's F10 supplemented with 10% FCS, 10% heat-inactivated horse serum, and 100 μg/ml Primocin (Invitrogen, San Diego, CA). After filtration of the cell suspension through nylon netting (pore size, 40 μm), the satellite cells were seeded in 35-mm plates. After 8–15 d of proliferation (37 C, 5% CO_2) at the end of which the cells align, the growth medium was replaced by the fusion medium, DMEM supplemented with 10% FCS, 1 mM L-glutamine, 1% penicillin-streptomycin, and 10 μg/ml insulin to induce the fusion of myoblasts into myotubes. This medium, which will be referred to as basal medium for sake of simplicity, was changed every 2 or 3 d, and differentiation was allowed to continue for 6 d (time required to obtain mature myotubes with characteristic elongated and multinucleated morphology) before the experimentation period.

At the time zero point, recombinant murine or human cytokines [all from Peprotech, except for murine TNFα (R&D Systems)] and/or other agents [L-NAME, *N*-acetylcysteine (NAC; Merck, VWR International, Zaventem, Belgium), rosiglitazone (Ro; a kind gift from GlaxoSmith-Kline Pharmaceuticals, Worthing, UK)] were added to the basal medium for up to 48 h. The cytokines were added to the medium every 24 h; the concentrations used were similar to those reported by others (21) and devoid of overt cytotoxicity. At the end of the culture, aliquots of medium were saved and stored at −80 C for subsequent nitrite assay, and the cells rinsed twice in PBS before RNA isolation.

RNA extraction and real-time quantitative PCR (RTQ-PCR)

RNA was isolated from muscles and cultured cells by using an acid guanidinium-thiocyanate-phenol-chloroform mixture (22) and TriPure isolation reagent (Roche Diagnostics, Vilvoorde, Belgium), respectively. Two micrograms of total RNA were reverse transcripted using oligo (dT) primers and Superscript II Rnase H^- reverse transcriptase (Invitrogen, Life Technologies). RTQ-PCR primers were designed (Primer Express software, Applied Biosystems, Foster City, CA) for mouse ApN, GLUT4, heme oxygenase-1 (HO-1), inducible NO synthase (iNOS), cyclophilin (Cyclo), human ApN, and hypoxanthine guanine phosphoribosyl transferase (HPRT) (Table 1). Then 120 ng total RNA equivalents were amplified with iQ SYBR green supermix (Bio-Rad Laboratories, Nazareth, Belgium) containing 300 nM of each specific primer using iCycler iQ real-time PCR detection system (Bio-Rad). The threshold cycles (Ct) were measured in separate tubes and in duplicate. The identity and purity of the amplified product was checked by electrophoresis on agarose minigels and analysis of the melting curve carried out at the end of amplification. To ensure the quality of the measurements, each plate included a negative control for each gene.

The ΔCt values were calculated in every sample for each gene of interest as followed: $Ct_{\text{gene of interest}} - Ct_{\text{reporter gene}}$ with Cyclo or HPRT as the reporter gene (mRNA of reporters remained stable throughout the experiments). Relative changes in the expression level of one specific gene (ΔΔCt) were calculated as ΔCt of the test group minus ΔCt of the

TABLE 1. Murine (m) or human (h) gene sequences used as forward and reverse primers for RTQ-PCR

Gene	Sense primer	Reverse primer	Amplicon (bp)
mApN	GCA GAG ATG GCA CTC CTG GA	CCC TTC AGC TCC TGT CAT TCC	101
mCyclo	AAC CCC ACC GTG TTC TTC	TGC CTT CTT TCA CCT TCC C	364
mGLUT4	GCC CCA CAG AAG GTG ATT GA	AGC GTA GTG AGG GTG CCT TG	101
miNOS	CCA GTT TTT GAT CCT CAC GTG	TGA CCT GAA AGA GGA AAA GGA C	200
mHO-1	ACA GCA TGT CCC AGG ATT TGT C	AAG GAG GCC ATC ACC AGC TT	142
hApN	TGA AGG ATG TGA AGG TCA GCC	TCC CCA TAC ACC TGG AGC C	151
hHPRT	TCA GGC AGT ATA ATC CAA AGA TGG	AGT CTG GCT TAT ATC CAA CAC TTC	83

Last column indicates the length of amplicon (bp).

control group and then presented as $2^{-\Delta\Delta Ct}$ (23). The PCR efficiency equaled 1 for each gene studied.

Quantification of ApN

ApN concentrations were determined by a commercially available kit (RIA mouse adiponectin kit; Linco Research, St. Charles, MO) in mouse tibialis anterior muscles or myotubes homogenized in 1 ml $NaHCO_3$ buffer [20 mM (pH 7.0)] containing saccharose (250 mM), NaN_3 (5 mM), phenylmethylsulfonyl fluoride (100 μM), aprotinin (10 μg/ml), and leupeptin (10 μg/ml) (24). Samples (50 μl) were run in duplicate. The intraassay variability was 3.73 and 4.11% at concentrations of 3 and 8 ng/ml, respectively. The interassay variability was 8.24 and 6.56% at the same concentrations. Protein concentrations were measured in each sample by the Bradford method, thereby allowing determination of total protein content in muscle or cultured myotubes.

Nitrite determination

Nitrite (NO_2^-) is a stable end product used extensively as an indicator of NO production. In our experimental conditions, NO_2^- accumulation was assayed by the Griess reaction (25). For plasma samples, proteins were first precipitated and nitrate reduced to NO_2^- as described (26). NO production was thus measured as nitrate plus nitrite (NO_x) concentrations. Protein-free plasma samples (250 μl) or culture medium (400 μl) was mixed with four times the amount of Griess reagent (1% wt/vol sulfanilamide, 0.1% wt/vol naphthylethylenediamine, 2.5% vol/vol H_3PO_4). Samples were incubated at room temperature for 10 min and absorbance was subsequently read at 543 nm using a spectrophotometer. NO_2^- concentrations were calculated in comparison with a sodium nitrite ($NaNO_2$) standard curve.

Results presentation and statistical analysis

Results are the means ± SEM for indicated numbers of individual mice (*in vivo* study) or separate experiments (*in vitro* studies). Ranges for gene expression levels were presented in each figure as $2^{-(\Delta\Delta Ct \pm SEM)}$, where SEM is calculated from the ΔΔCt values (23; user bulletin no. 2 Applied Biosystems, http://www.appliedbiosystems.com/search/ and search for 777802–001).

Comparisons between two conditions were made using two-tailed unpaired Student's *t* test. Comparisons of at least three conditions were carried out by ordinary or repeated ANOVA followed by the Newman Keuls (comparison of all pairs) or Dunnett's tests (all *vs.* control) as appropriate. Statistical analysis for gene expression levels was performed on the ΔΔCt values. Differences were considered statistically significant at $P < 0.05$.

Results

Induction of ApN in muscle in vivo

Basal ApN gene expression was stable and low (Ct ~27) in tibialis anterior muscle of saline-injected control mice (Fig. 1A and data not shown). Injection of a small dose of LPS caused an approximately 10-fold rise in ApN mRNAs at 24 h (Fig. 1A). Concomitantly, ApN levels increased by approximately 70% in muscle of LPS-injected mice, compared with control animals (Fig. 1B, data in nanograms per milligram protein). Quantitatively similar results were obtained when data were expressed as nanograms per milligram muscle (not shown).

Because LPS is a strong inducer of several proinflammatory cytokines, we also tackled the effects of a cytokine mix *in vivo*. Injection of IFNγ combined with TNFα tended to elevate ApN mRNAs in mouse tibialis anterior at 24 h (2.2-fold *vs.* control mice; n = 5 animals per group, $P = 0.10$).

Induction of ApN mRNA in C_2C_{12} cells

To further investigate the mechanisms by which LPS inflammation induced ApN gene expression in muscle, we used an *in vitro* approach. We first attempted to reproduce ApN induction in C_2C_{12} myotubes cultured for 48 h with various proinflammatory cytokines (IL-1β, IL-6, IFNγ, or TNFα). None of the cytokines used alone was able to modify ApN gene expression. Some combinations were tested. IFNγ together with IL-1β failed to induce ApN mRNAs. However, in agreement with the *in vivo* study, the combination of IFNγ and TNFα was effective. This combination markedly in-

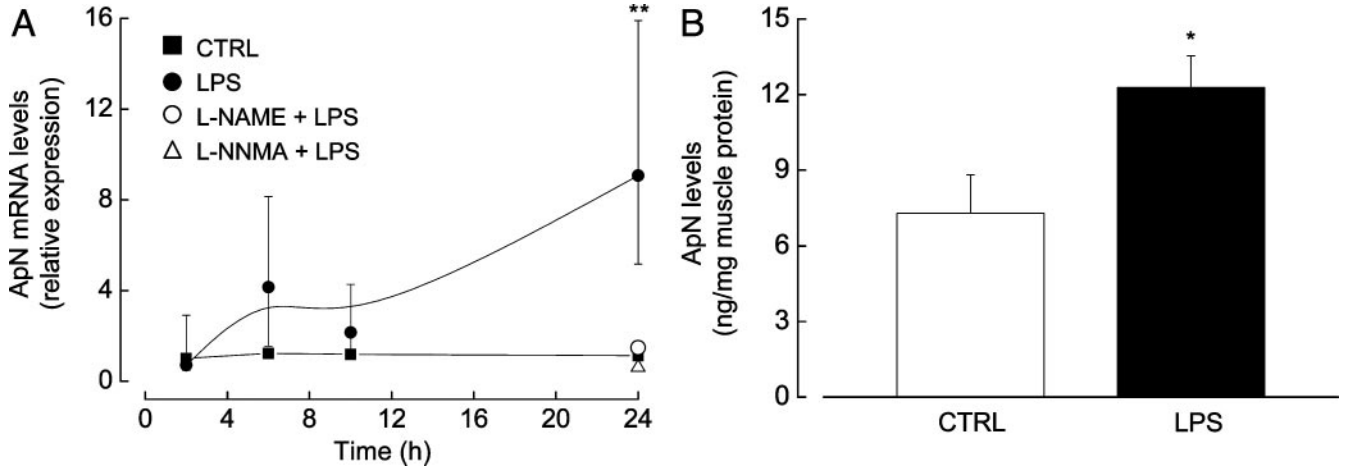

FIG. 1. Effect of LPS injection on ApN mRNA (A) and tissue levels (B) in mouse tibialis anterior muscle. Male mice were injected ip with 25 μg LPS dissolved in saline, whereas control (CTRL) mice received the vehicle only. In some experiments, mice were treated with inhibitors of the NO synthases, L-NAME (10 mg/kg ip) or L-NNMA (20 mg/kg ip) 30 min before LPS injection. The animals were killed 2, 6, 10, or 24 h after the injection. Muscles were collected at each time point (mRNA) or only at the last time point (24 h; tissue ApN). Muscles were flushed with saline before sampling for tissue ApN content (to avoid contamination by circulating ApN). mRNA levels were quantified by RTQ-PCR and are presented as relative expression, compared with control values at 24 h. ApN levels were measured in tissue homogenates by RIA and expressed as nanogram per milligram protein. Results are the means ± SEM for nine CRTL, nine LPS, five L-NAME + LPS, and five L-NNMA + LPS mice at 24 h and three mice for each CRTL or LPS group at other time points (A) or six mice per group at 24 h (B). SEM within the symbols were omitted for sake of clarity in A. *, $P < 0.05$, **, $P < 0.01$ *vs.* time-matched controls.

creased ApN mRNAs, suggesting a synergistic rather than an additive action of both cytokines (Fig. 2A). ApN protein levels also rose significantly in treated myocytes (Fig. 2B).

The time course of mRNA induction by this mix is shown in Fig. 3A. Under basal (control) conditions, ApN mRNA levels remained stable and low in C_2C_{12} myotubes cultured for up to 48 h. In contrast, IFNγ combined with TNFα (both used at 5 ng/ml concentrations) doubled ApN mRNA after 24 h, and this stimulation was further amplified after 48 h.

Stimulation of ApN gene expression was next examined in myotubes cultured with a fixed concentration of IFNγ (10 ng/ml) and increasing concentrations of TNFα for 48 h. Under these conditions, the stimulation was detectable at TNFα concentrations above 1 ng/ml, maximal at 2.5 ng/ml, and then plateaued (Fig. 3B). Likewise, in the presence of TNFα at a fixed concentration (2.5 ng/ml), ApN mRNAs rose dose-dependently with increasing concentrations of IFNγ (Fig. 3C).

Mechanisms of ApN induction

We first hypothesized that excessive production of reactive oxygen species by cytokines could mediate ApN gene induction (27). To test this assumption, we examined whether NAC, an antioxidant, was able to inhibit the effect of the cytokine mix. Figure 4A clearly shows that NAC failed to prevent ApN mRNA induction by cytokines. Yet in our system, NAC exerted its expected antioxidant action as shown by its inhibitory effect on HO-1 mRNA, an enzyme induced in response to oxidative stress (28) (Fig. 4B). Thus, NAC largely prevented cytokine-induced accumulation of HO-1 mRNA levels. Moreover, NAC used at the same concentration (10 mM) fully reversed the potent induction of HO-1 expression produced by 100 μM H_2O_2 (data not shown).

We next examined whether NO production by cytokines was involved in ApN induction. Unlike the other experimental conditions, IFNγ combined with TNFα for 48 h potently enhanced NO release in the medium of C_2C_{12} cells, through marked (~300-fold) up-regulation of iNOS mRNA (Fig. 5, A and E) (29). L-NAME, a competitive inhibitor of NO synthases, abrogated both cytokine-induced NO production and ApN mRNA induction without affecting iNOS mRNA induction (Fig. 5, C–E). The time course of iNOS up-regulation further supports a role for NO in ApN induction (Fig. 5B). iNOS mRNA levels were already significantly increased in myotubes after 8 h of culture in the presence of the cytokine mix and reached a maximum at approximately 24 h, whereas ApN induction was detectable only from 24 h onward. Hence, iNOS induction clearly preceded ApN induction. In this context, we measured plasma NO_x concentrations in mice injected with LPS or saline and observed a similar finding. As expected (26), NO_x levels were significantly higher in LPS mice from 2 h onward, reached a peak at 6 h [28 ± 0.1 μM (LPS mice) *vs.* 8 ± 0.1 μM (saline mice), n = 3 at each point as in Fig. 1A; $P < 0.01$], and then returned to control levels at 24 h (not shown). Accordingly, the *in vivo* peak of NO production also preceded ApN up-regulation in tibialis anterior muscle. Eventually, in the light of the results obtained in C_2C_{12} cells, we treated mice with inhibitors of the NO synthases (either L-NAME or L-NMMA) 30 min before LPS injection. As expected, this treatment fully prevented LPS-induced ApN mRNA up-regulation (Fig. 1A).

Because the thiazolidinedione Ro may suppress LPS- and IFNγ-target genes in a PPARγ-dependent manner in macrophages (30), we tested whether this antidiabetic drug could exert similar properties in C_2C_{12} cells. As shown in Fig. 6A, 10 μM Ro for 48 h did not affect ApN mRNA levels when tested either alone or in combination with the cytokine mix. Thus, unlike in macrophages, Ro failed to inhibit induction of cytokine target genes (*i.e.* ApN) in myotubes. However, Ro exerted its expected stimulatory effect on the insulin-sensitive glucose transporter gene whether the cytokine mix was present or not, as shown by the 2- to 3-fold increase of GLUT4 mRNAs in C_2C_{12} cells (Fig. 6B) (31).

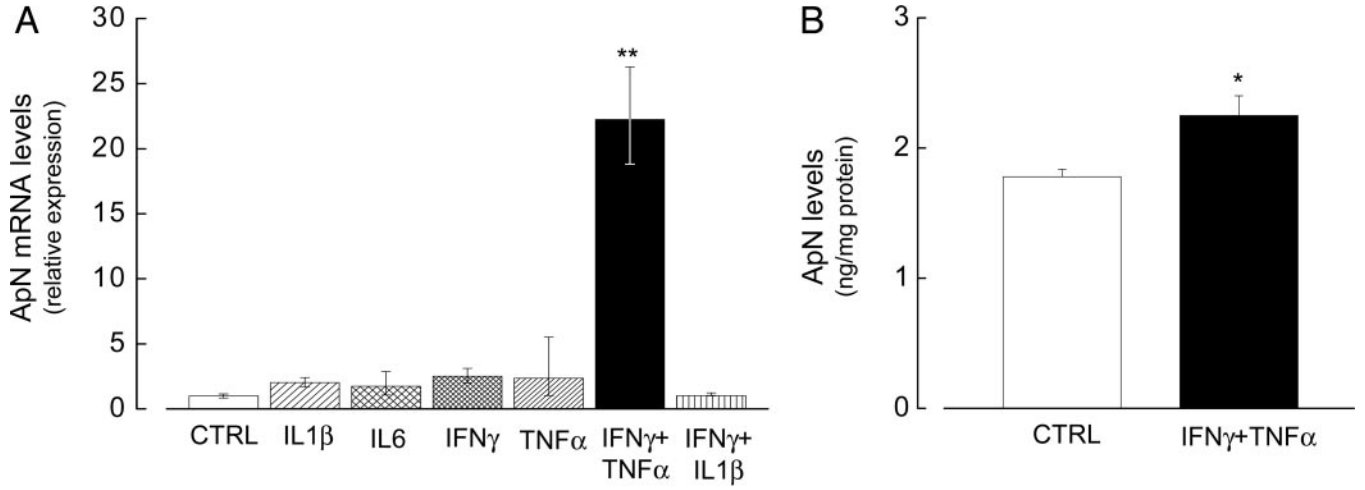

FIG. 2. Effects of cytokines on ApN mRNA (A) and protein levels (B) in C_2C_{12} myotubes. Differentiated C_2C_{12} cells were cultured for 48 h without (control, CTRL) or with the indicated cytokine(s) (each used at a 5 ng/ml concentration). A, mRNA levels were quantified by RTQ-PCR and are presented as relative expression, compared with control values. B, ApN levels were measured in cell homogenates by RIA and expressed as nanogram per milligram protein. Results are the means ± SEM for four to six independent experiments. *, $P < 0.05$ *vs.* CTRL; **, $P < 0.01$ *vs.* all other conditions.

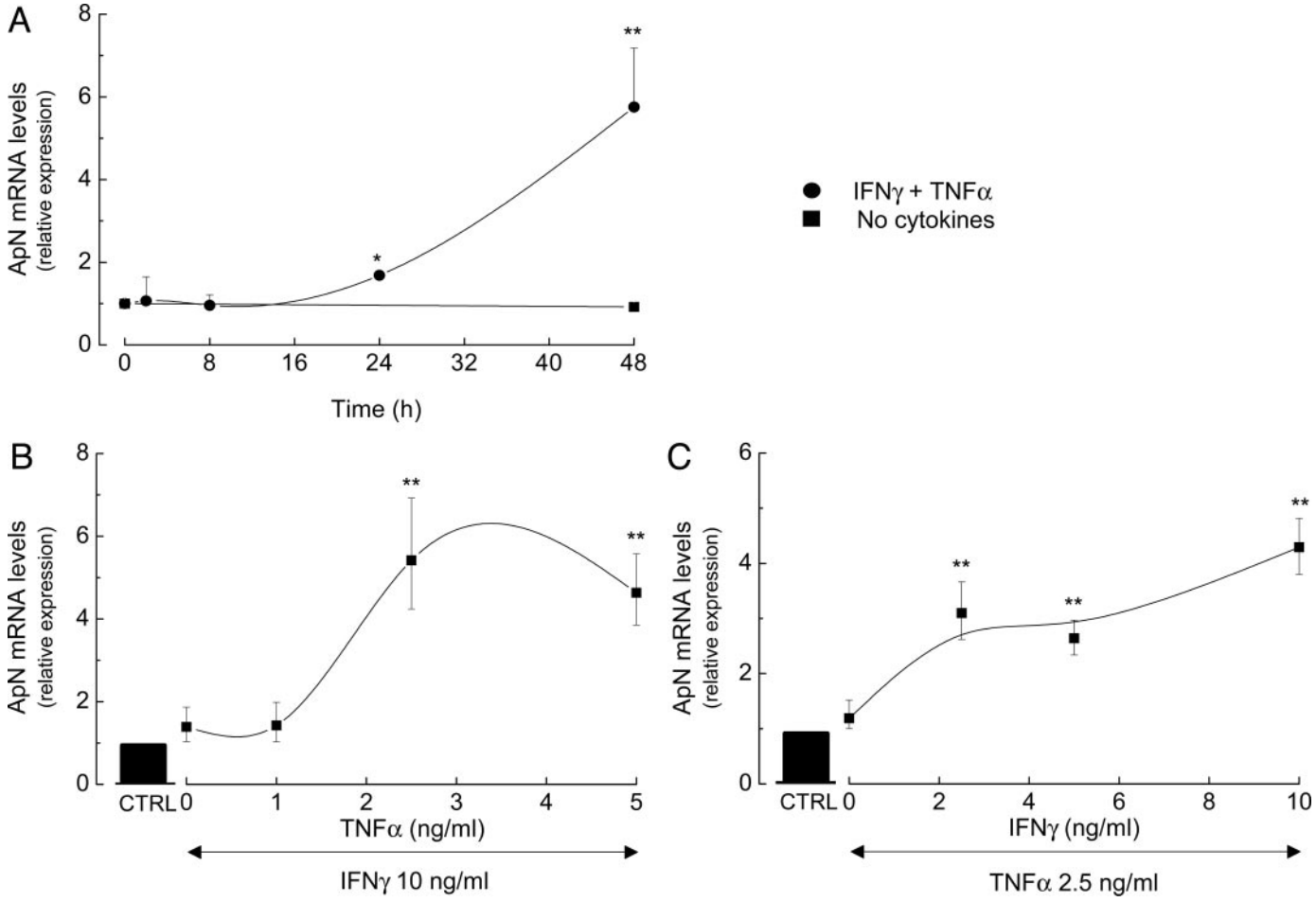

FIG. 3. Time- and dose-dependent effects of cytokine combination on ApN gene expression in C_2C_{12} myotubes. A, Induction of ApN in C_2C_{12} cells cultured for up to 48 h without (■) or with (●) IFNγ (5 ng/ml) and TNFα (5 ng/ml). B and C, Dose-dependent effects of cytokine combination on ApN mRNA levels in C_2C_{12} cells cultured for 48 h with a fixed concentration of IFNγ (10 ng/ml) and increasing concentrations of TNFα (B) and vice versa, the concentration of TNFα being fixed at 2.5 ng/ml (C). mRNA levels were quantified by RTQ-PCR and are presented as relative expression, compared with control values [*i.e.* no cytokines at 0 h (A) or over the 48-h culture (CTRL, B and C)]. Results are the means ± SEM for four experiments. *, $P < 0.05$, **, $P < 0.01$ *vs.* respective controls.

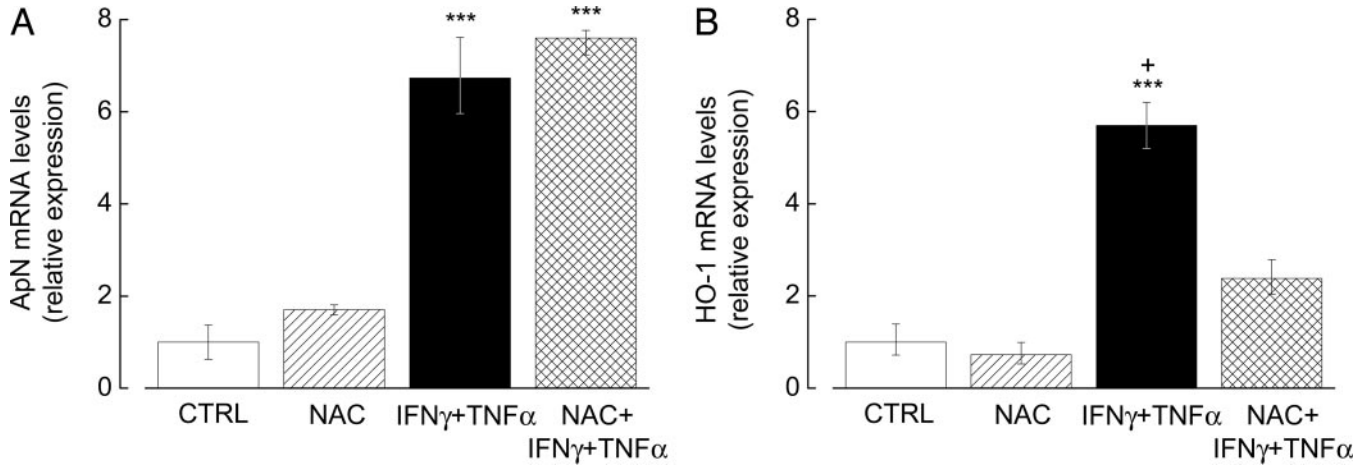

FIG. 4. Influence of NAC on cytokine induction of ApN and HO-1 gene expression in C_2C_{12} myotubes. C_2C_{12} cells were cultured for 48 h in the absence (control, CTRL) or presence of IFNγ and TNFα (both used at 5 ng/ml concentrations) and/or NAC (10 mM). The antioxidant was added 30 min before the cytokines. mRNA levels were quantified by RTQ-PCR and are presented as relative expression, compared with control values. Results are the means ± SEM for four independent experiments. ***, $P < 0.001$ *vs.* controls and NAC alone; $^{+}$, $P < 0.05$ *vs.* NAC + cytokine mix.

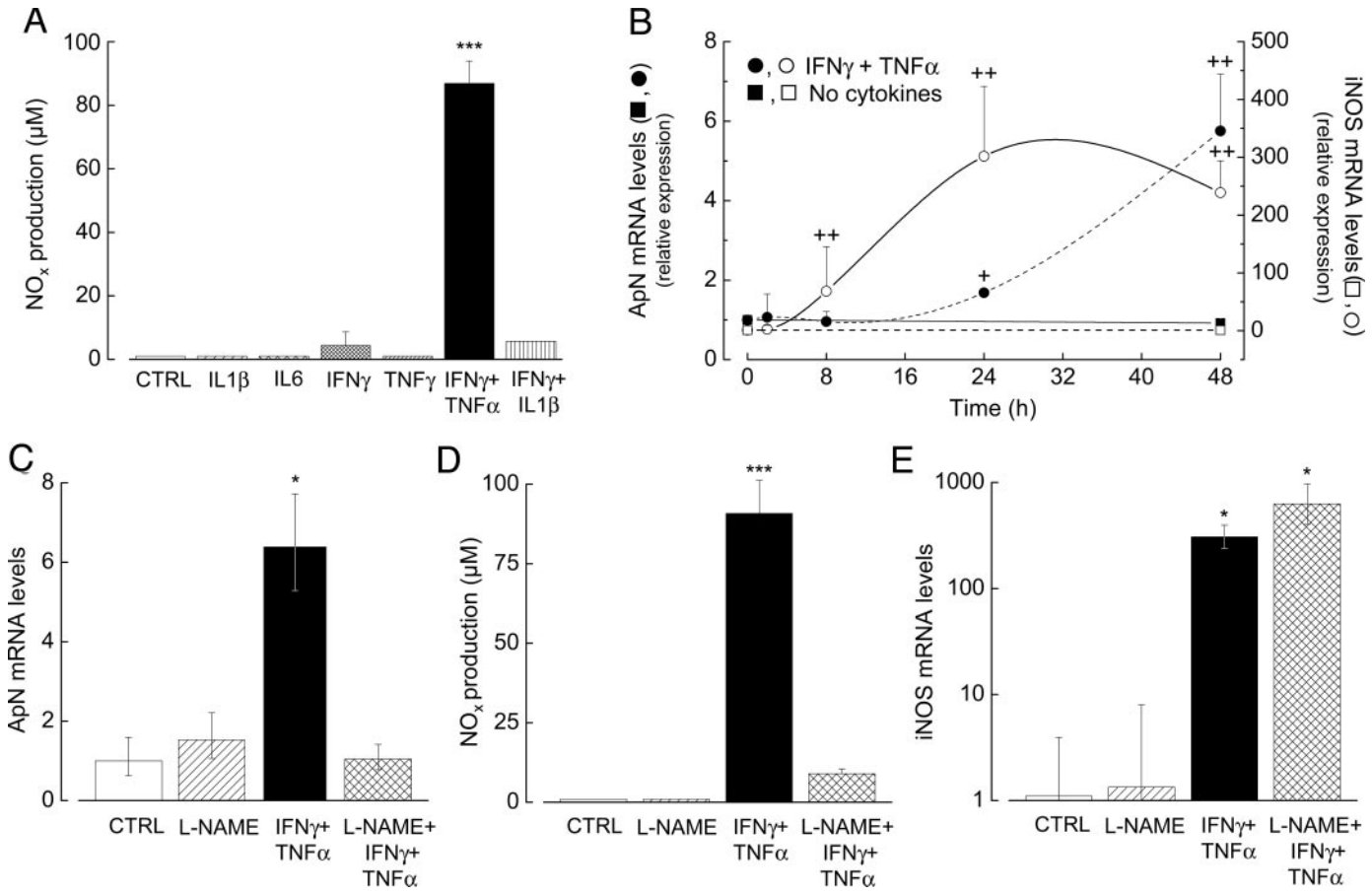

FIG. 5. Role of NO on cytokine-induced up-regulation of ApN in C_2C_{12} myotubes. C_2C_{12} cells were cultured for 48 h in the absence (control, CTRL) or with the indicated cytokine(s) (each used at a 5 ng/ml concentration). A, NO release by C_2C_{12} myotubes in the presence of various cytokines. NO_x concentrations were measured in medium of the same experiments as those depicted in Fig. 2A. B, Induction of ApN (■, ●) or iNOS mRNA (□, ○) in C_2C_{12} cells cultured for up to 48 h without (■, □) or with (●, ○) IFNγ (5 ng/ml) and TNFα (5 ng/ml). These experiments are the same as those shown in Fig. 3A, in which only ApN was presented. C–E, Influence of L-NAME on cytokine induction of ApN mRNA, NO production, or iNOS gene expression. C_2C_{12} cells were cultured for 48 h in the absence (control, CTRL) or with the cytokine mix described above and/or L-NAME, an inhibitor of NO synthases (5 mM). NO_x concentrations were measured in medium as supra. mRNA levels were quantified by RTQ-PCR and are presented as relative expression, compared with control values [*i.e.* no cytokines over the 48 h-culture (CTRL, in A, C, and E) or at 0 h (B)]. Results are the means ± SEM for four independent experiments. *, $P < 0.05$; ***, $P < 0.001$ *vs.* all conditions without *asterisks*; $^{+}$, $P < 0.05$; $^{++}$, $P < 0.01$ *vs.* respective controls.

Induction of ApN mRNA in human mature myotubes

Finally, we showed that ApN mRNA induction by cytokines was reproduced in humans *in vitro*. As depicted in Fig. 7, the combination of IFNγ and TNFα enhanced ApN mRNA abundance in primary cultures of human skeletal muscle cells.

Discussion

In this study, we showed that the adipose hormone, ApN, may be induced in the skeletal muscle of mice injected with LPS. Endotoxin inoculation is known to cause an acute inflammatory state and trigger the production of numerous cytokines including IFNγ, IL-1β, IL-6, or TNFα in classical immune tissues as well as nonimmune tissues such as skeletal muscle (32, 33). Admittedly, this tissue is composed of several cell types, and we did not prove that ApN was specifically expressed within myocytes. However, we then demonstrated that a combination of cytokines was able to reproduce ApN induction in cultured murine and human myotubes. Importantly, the concentrations of cytokines used *in vitro* were similar to those reached *in vivo* in the plasma of LPS-injected mice (19, 34, 35).

We next characterized the mechanisms responsible for ApN induction mostly *in vitro* but also *in vivo*. Several pieces of evidence suggest that NO mediates ApN induction by cytokines in myotubes or muscle. First, ApN was induced *in vitro* exclusively in the experimental conditions that stimulated NO production. In this respect, only the combination IFNγ + TNFα was efficient in up-regulating both ApN expression and NO synthesis. Second, iNOS mRNA induction clearly preceded ApN induction in myotubes. This is consistent with the *in vivo* data. Plasma NO_x concentrations were higher in mice injected with LPS than in saline mice and reached a peak 6 h after treatment, thereby also preceding ApN up-regulation in tibialis anterior muscle. Third, preventing NO production by using inhibitors of the NO synthases, L-NAME or L-NNMA, suppressed the inductive effect of the cytokine mix or LPS both *in vitro* and *in vivo*.

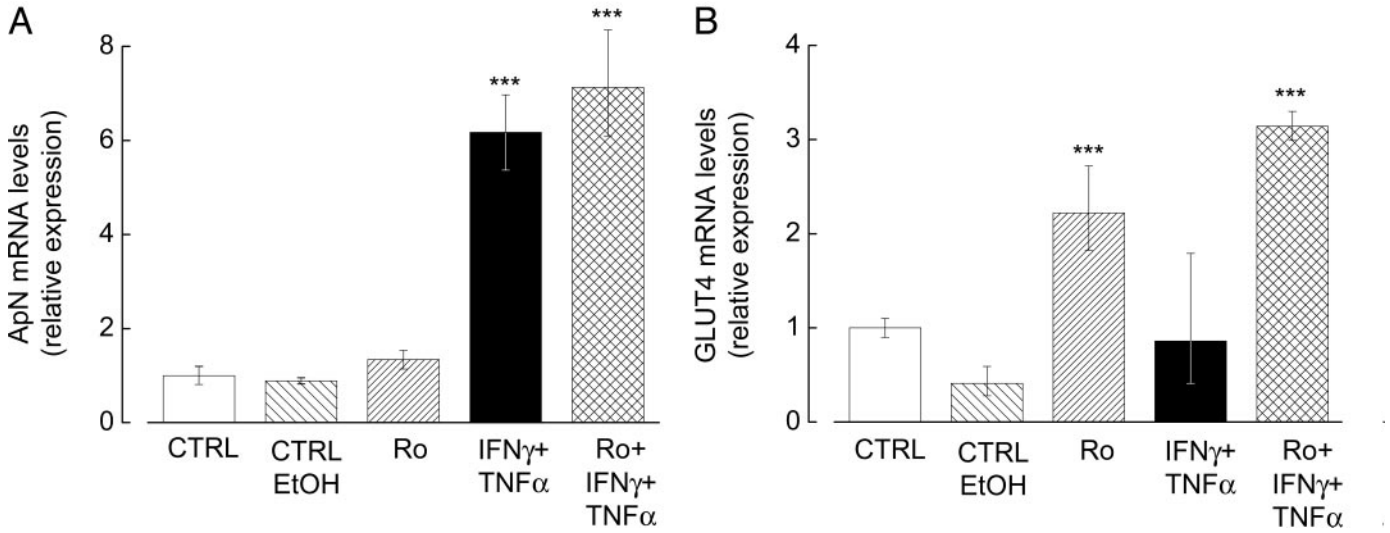

FIG. 6. Effect of Ro on ApN and GLUT4 gene expression in C_2C_{12} myotubes stimulated by cytokines. C_2C_{12} cells were cultured for 48 h in the absence (control, CTRL) or presence of IFNγ and TNFα (both used at 5 ng/ml concentrations) and/or Ro (10 μM). mRNA levels were quantified by RTQ-PCR and are presented as relative expression, compared with control values (CTRL). A second control with Ro vehicle (ethanol, EtOH) was also used. Results are the means ± SEM for four independent experiments. ***, $P < 0.001$ *vs.* respective controls.

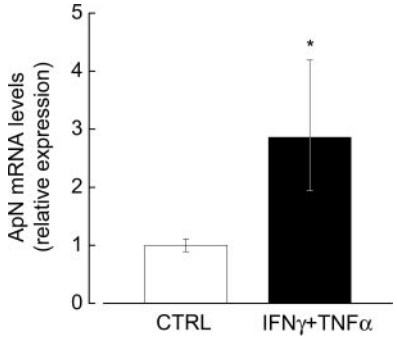

FIG. 7. Cytokine induction of ApN mRNA in primary cultures of human skeletal muscle cells. Mature myotubes were cultured for 48 h in the absence (control, CTRL) or presence of IFNγ and TNFα (both used at 5 ng/ml concentrations). mRNA levels were quantified by RTQ-PCR and are presented as relative expression, compared with control values (CTRL). Results are the means ± SEM for three independent cultures, each performed with tissue obtained from a different patient. *, $P < 0.05$ *vs.* controls.

To date, the adipocyte has been considered the almost exclusive site of ApN production (17). Our study is one of the very few that deals with ApN regulation in nonadipose cells or tissues. In mouse liver, ApN was found to be elevated after carbon tetrachloride administration, a finding that is not surprising because hepatic injury may result in steatosis (36). More recently it has been reported that ApN could be induced in human myotubes exposed to an adiponectin-containing human embryonic kidney 293 cell culture supernatant (18). We demonstrate here that ApN can be up-regulated both in muscle *in vivo* and myotubes *in vitro* in response to proinflammatory cytokines. In many respects, ApN regulation in muscle seems to be fully distinct, if not opposite, from that described in adipose tissue. First, in contrast to the up-regulation found in muscle, LPS injection down-regulated ApN mRNA levels of mouse adipose tissue (Bauche, I., and S. M. Brichard, unpublished data). Second, TNFα also inhibited ApN production in cultured adipocytes (37–39), whereas this cytokine induced ApN in C_2C_{12} cells incubated in the presence of IFNγ. Third, ApN regulation by PPARγ agonists is also quite different in the two tissues. Thiazolidinediones, potent adipogenic agents used as antidiabetic drugs, are strong inducers of ApN gene expression in adipose tissue (38, 40, 41) but had no effect on ApN production in myotubes. This lack of effect also enables us to ensure that ApN expression in myotubes did not result from contamination of the myotube cultures with adipocytes or from transdifferentiation of myotubes into adipocytes.

It is worth noting that the maximum ApN mRNA content reached in muscle remained lower than that obtained in adipose tissue. In our *in vivo* experiment, 24 h after LPS injection, ApN mRNA levels in tibialis anterior muscle were 2 orders of magnitude lower than those measured in epididymal adipose tissue (our unpublished data). Yet the local efficacy of ApN on myocytes might still be high because this adipocytokine could directly act on these cells via autocrine or paracrine mechanisms. In addition, ApN may undergo some posttranslational modifications (*e.g.* a proteolytic cleavage leading to the globular form) that could further raise its local potency (4).

The physiological relevance of this overexpression of ApN in response to inflammatory injury in muscle is still under investigation. On one hand, ApN exerts antiinflammatory properties on several cell types or tissues (endothelium, macrophages, and pancreatic β-cells) (10, 11, 13, 31). In these cells, it controls early events of inflammation but also late events by preventing immune responses from continuing chronically (13). Thus, induction of ApN in muscle may be viewed as a protective mechanism against excessive and deleterious inflammatory reactions. On the other hand, ApN up-regulation in muscle could also be a useful means to meet the extra energy needs of inflammatory processes. This may be achieved from the ability of ApN to stimulate fatty acid oxidation and glucose transport in skeletal muscle (2, 3, 7).

In conclusion, our data provide evidence that adiponectin is up-regulated *in vivo* and *in vitro* in human and rodent myotubes in response to inflammatory stimuli. The underlying mechanisms seem to involve a NO-dependent pathway. This overexpression may be viewed as a local antiinflammatory protection and a way to deliver extra energy supplies during inflammation.

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Address all correspondence and requests for reprints to: S. M. Brichard, Unité d'Endocrinologie et Métabolisme, UCL/ENDO 5530-Avenue Hippocrate, 55, B-1200 Brussels, Belgium. E-mail: brichard@endo.ucl.ac.be.

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