Tumour necrosis factor alpha regulates iron transport and transporter expression in human intestinal epithelial cells

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Abstract TNFα has dramatic effects on iron metabolism contributing to the generation of hypoferraemia in the anaemia of chronic disease. Interestingly, TNFα is also synthesised and released within the intestinal mucosa, suggesting that this pro-inflammatory cytokine may play a role in regulating dietary iron absorption. To investigate this possibility, we stimulated intestinal Caco-2 cells with TNFα (10 ng/ml). In TNFα-treated cells, apical iron uptake was significantly decreased and this was accompanied by a reduction in divalent metal transporter protein and mRNA expression. Our data suggest that TNFα could regulate dietary iron absorption and that the apical transport machinery is the target for these actions.

Keywords: Divalent metal transporter; Iron regulated transporter; Iron transport; Caco-2 cells

1. Introduction

Iron is an essential nutrient in the human diet. Recently, a number of the key components of the iron transport pathway in intestinal enterocyte have been elucidated (reviewed in [1]). However, the fine control of absorption remains unclear. It has been proposed that overall regulation of dietary iron assimilation is mediated via signals from three distinct compartments that reflect dietary composition, the status of the body iron stores, and the metabolic requirements – especially for the production of new red blood cells. These signals are generally referred to as the *dietary*, *stores*, and *erythroid* regulators, respectively.

Recently, hepcidin, a 20–25 amino acid peptide produced in the liver [2,3], has been proposed as one possible mediator of the stores and erythroid regulatory pathway. However, it seems likely that a number of other humoral factors may also be important in determining the amounts of iron absorbed from the diet, including transferrin saturation [4], and the plethora of cytokines that regulate body iron utilisation [5]. In the present study, we have focussed on the role of one of these cytokines tumour necrosis factor alpha (TNFα) in regulating intestinal iron absorption. TNFα is a pro-inflammatory cytokine and has been implicated in the hypoferraemia associated with the anaemia of chronic disease [6,7]. Moreover, both dietary [8] and circulatory iron [9] can influence TNFα production. The synthesis of TNFα by cells residing within the intestinal mucosa in response to high iron intakes [8] raises the intriguing possibility that this cytokine might play a role as a local dietary regulator of intestinal iron absorption.

To investigate the possible intestinal interaction between TNFα and iron transport, we have used the human intestinal Caco-2 cell line. Caco-2 cells differentiate spontaneously in culture taking on an enterocyte-like phenotype expressing a number of small intestinal brush border enzymes and nutrient transporters [10,11]. Importantly for our studies, Caco-2 cells also express the key genes and proteins involved in iron transport, including divalent metal transporter (DMT1), iron regulated transporter (IREG1), ferritin, Dcytb and hephaestin [12–16] and are generally regarded as the best available intestinal cell model for studying the mechanisms associated with vectorial iron transport.

In the present study, cells were stimulated with TNFα for 72 hours and used to measure changes in iron uptake and efflux, as well as alterations in the expression of the transporters DMT1 and IREG1 that are responsible for iron transport across the apical and basolateral membranes, respectively, of intestinal enterocytes [17–21].

2. Materials and methods

2.1. Cell culture

Caco-2 cells (the TC7 subclone used in our previous studies [12–15]) were cultured in a 95% air/5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 20% heat-inactivated foetal bovine serum. All experiments were carried out on cells between passage numbers 35 and 42. For experiments, cells were seeded at a density of 1×10^4 cells/cm^2 onto Transwell inserts (Costar UK, Buckinghamshire, UK) and used 21 days later at which time they were fully differentiated [10,11,14]. To determine the effects of TNFα on iron transport and transporter expression, cells were cultured in the presence or absence of TNFα (10 ng/ml) added to the

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Abbreviations: TNFα, tumour necrosis factor alpha; DMT1, divalent metal transporter; IREG1, iron regulated transporter; IRE, iron responsive element; IRP, iron regulatory protein; UTR, untranslated region; RT-PCR, reverse transcription polymerase chain reaction; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; MES, 2-(*N*-morpholino)ethanesulphonic acid

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basolateral chamber of the Transwell plate for the final 72 h of the culture period.

2.2. Iron uptake by Caco-2 TC7 cell monolayers

The measurement of iron uptake by Caco-2 TC7 cells has been described previously [12]. Briefly, transepithelial pH gradients were produced using *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES)-buffered salt solution (pH 7.5; 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES) in the basolateral chamber, with 2-(*N*-morpholino)ethanesulphonic acid (MES)-buffered salt solution (pH 5.5 – substituting MES for HEPES) added to the apical chamber. Uptake was initiated by the addition of 10 μM Fe^{2+} complexed with 1 mM ascorbic acid (freshly prepared prior to the start of each experiment) and 37 kBq/ml $^{55}FeCl_3$ to the apical chamber. Previous studies have shown that a 10–50-fold excess of ascorbate was sufficient to maintain maximal levels of Fe(II) in solution [22]. The reaction was terminated after 15 min, which is within the linear portion of the iron uptake curve [12] and cell monolayers were washed 3 times in ice cold transport buffer containing a 10-fold excess of iron, solubilised overnight in 200 mM NaOH, and finally subjected to scintillation counting to determine iron uptake. In order to assess basolateral efflux, uptake was allowed to reach equilibrium (after 60 min [12]). Cells were subsequently placed into fresh uptake buffers and incubated for a further 60 min. The level of iron present in the basolateral medium at the end of this period was determined by scintillation counting.

2.3. Western blot analysis

Following removal of culture medium, cell monolayers were washed twice in phosphate buffer and harvested from the insert using a cell scraper. Cells were pelleted by centrifugation at $750 \times g$ for 5 min and resuspended in 250 μl PBS (containing 0.5 mM PMSF, 0.2 mM benzamidine and 0.02% NaN_3) and homogenised at full power with an UltraTurrax homogeniser for 2×30 pulses.

Cell homogenates (20 μg) were solubilised in sample buffer and subjected to SDS–PAGE. The proteins were transferred onto nitrocellulose (Hybond ECL – Amersham Pharmacia Biotech, Buckinghamshire, UK) and blocked overnight in phosphate buffer containing 0.05% Tween 20 and 1% fat free milk. The nitrocellulose was incubated for 2 h at room temperature with commercially available polyclonal antibodies to DMT1 [both iron responsive element (IRE) and non-IRE isoforms] and IREG1 (ADI –TX, USA) or villin (Santa Cruz Biotechnology Inc., CA, USA). Following removal of the primary antibody, nitrocellulose filters were incubated with an HRP-linked secondary antibody, and cross reactivity visualised using ECL Plus and Hyperfilm ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Band densities were semi-quantified by densitometric analysis using Scion Image software (Scion Corporation, MD, USA).

2.4. RT-PCR

Total RNA was isolated from Caco-2 cells using Trizol reagent (Life Technologies, Paisley, UK) and stored at −70 °C in 75% ethanol until required. Reverse transcription polymerase chain reaction (RT-PCR) was performed in a single step reaction, using Ready-to-go RT-PCR Beads (Amersham Pharmacia Biotech) on total RNA samples (1 μg per tube) using the primer sequences and conditions described previously [15].

2.5. Cloning of DMT1 promoter and reporter gene studies

Approximately 1.6 kb of the DMT1 promoter was amplified by PCR using primers derived from published sequences [23,24] (sense primer: CAG ACGCGT GGT CCC CGA GTT TTG TTT TTT ACT TCG CAC; antisense, CAG CTCGAG TTA CCA GCT CCG CAA CCA CCT G). These primers included restriction sites for *Mlu*I and *Xho*I (underlined). The amplified DNA was digested and subcloned into the *Mlu*I-*Xho*I sites of pGL3 Basic vector (Promega, Southampton, UK), creating a transcriptional fusion with the luciferase gene. The construct, pGL3NR2S, was sequenced to verify its authenticity.

The pGL3NR2S construct was transfected into pre-confluent Caco-2 cells using $CaPO_4$ (ProFection, Promega, Southampton, UK) and the cells were cultured for a further 3 days. On day 3 post-transfection, the cells were switched to serum-free medium containing TNFα and cultured for a further 72 h prior to assay for changes in luciferase activity.

2.6. Data analysis

Data are presented as means ± S.E.M. Statistical analysis was carried out using SPSS statistics package and utilised one-way ANOVA followed by Tukey's post hoc test or Student's unpaired *t* test where appropriate. Differences were considered significant at $P < 0.05$.

2.7. Materials

Radiochemicals and materials for Western blotting were supplied by Amersham Pharmacia Biotech. Cell culture medium, heat-inactivated foetal bovine serum and plasticware were purchased from Invitrogen (Paisley, UK) unless stated. Reagents and plasmids for cell transfections and reporter gene assays were obtained from Promega, Southampton, UK. Human TNFα was purchased from Sigma-Aldrich (Poole, Dorset, UK). All other chemicals were of highest grade available and bought from Sigma, Merck or Fluka.

3. Results and discussion

It has been proposed that TNFα acts on a number of cell types to inhibit the release of iron, thereby contributing to hypoferraemia [25] and that this in turn is mediated by an increase in ferritin synthesis and subsequent iron sequestration [26,27]. The intestine may be a target tissue for this action. TNFα is released from intraepithelial lymphocytes residing within the intestinal mucosa following increased dietary iron intake [28] through a mechanism that is thought to involve HFE on the basolateral surface of the crypt epithelium [29] and results in iron deposition within intestinal enterocytes via a TNF receptor 2-dependent mechanism [8]. In an attempt to elucidate the effects of TNFα on intestinal iron transport and transporter expression, we have used human intestinal Caco-2 cells exposed to the cytokine for 72 h. The level of TNFα used in our study (10 ng/ml) was selected because it is in the middle of the range of TNFα concentrations that have been used in previous studies on nutrient transport in Caco-2 cells [30,31] and in earlier in vitro studies on iron metabolism [32,33].

As a starting point for the current investigation, we measured ferritin protein expression in Caco-2 cells. Consistent with data from other studies, stimulation of Caco-2 cell with TNFα significantly increased ferritin levels [26,27] (Fig. 1). To determine whether the changes in cellular ferritin had any consequences for functional iron transport across the Caco-2 cell monolayer, we measured both iron uptake across the apical membrane into the cells and efflux into the basolateral medium. Iron uptake (measured over 15 min when uptake was linear [12]) across the apical membrane was significantly decreased in Caco-2 cells treated with TNFα compared with untreated controls (Fig. 2(a)). In parallel experiments, uptake was allowed to proceed to equilibrium (60 min in these studies). At this time, uptake was not significantly different in the control and TNFα-treated groups (data not shown). Cells were placed in fresh medium and incubated for a further 60 min to determine iron efflux into the basolateral medium. In cells treated with TNFα, there was a small but significant decrease in iron efflux (Fig. 2(b)). The decrease in iron efflux (i.e., increased retention within the cell) could be explained by the increase in ferritin synthesis and subsequent iron sequestration. However, we could not rule out the possibility that TNFα was having direct effects on the efflux transporter IREG1. To investigate this latter possibility, IREG1 protein and mRNA levels in Caco-2 cells were measured using Western blotting and RT-PCR, respectively. Expression of the "housekeepers", villin protein and β-actin mRNA, were unaffected by treatment

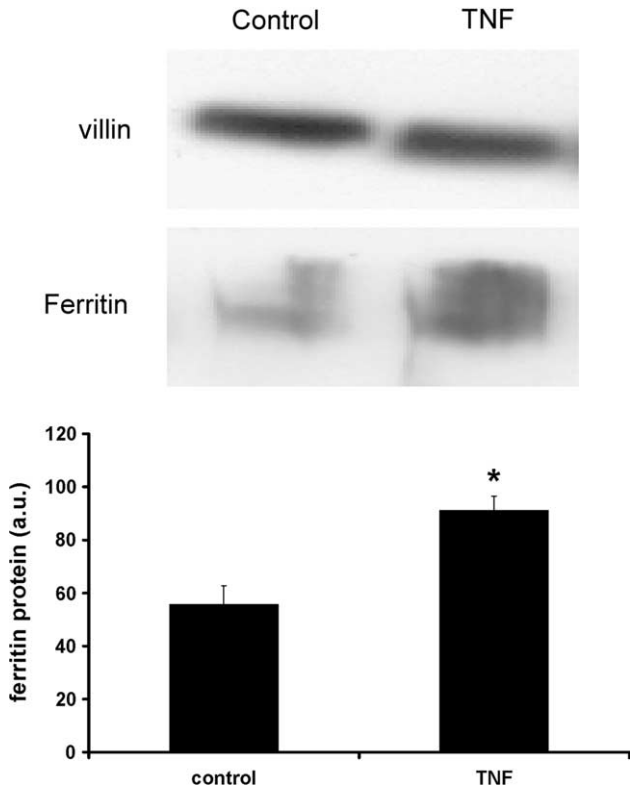

Fig. 1. Ferritin expression is increased in Caco-2 cells stimulated with TNFα. Caco-2 cells were grown for 21 days on Transwell inserts and were stimulated for the final 72 h of the culture period with TNFα (10 ng/ml), added to the basolateral medium. Cell homogenates were subjected to Western blotting using a polyclonal ferritin antibody (Sigma–Aldrich). Ferritin protein levels were significantly increased in TNFα-treated cells ($*P < 0.01$). Expression of the housekeeper protein villin in cell samples was not altered following cytokine treatment. Densitometry data are means ± S.E.M. of three separate experiments.

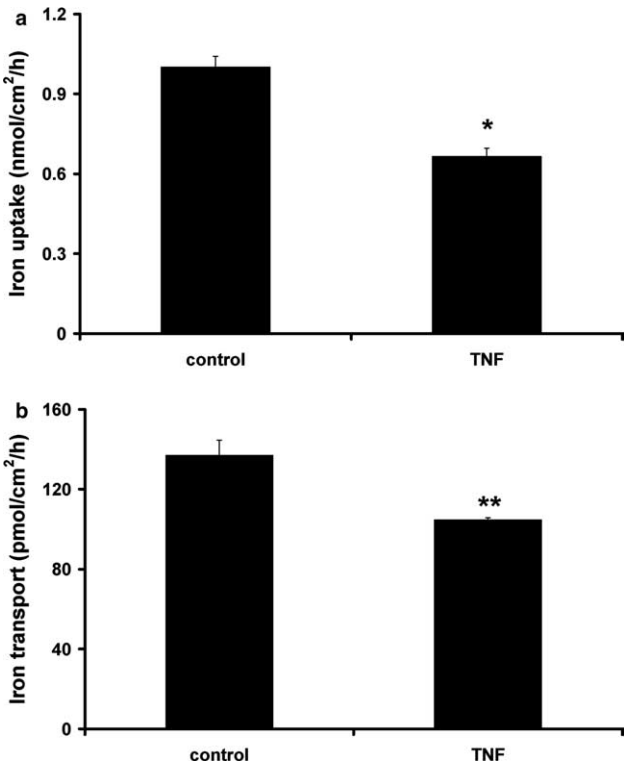

Fig. 2. Effect of TNFα on iron transport across Caco-2 cell monolayers. (a) Iron uptake across the apical membrane of Caco-2 cell stimulated with TNFα was determined over a 15 min time-course – we have previously shown that uptake is still linear at this time [10]. Uptake was significantly decreased in TNFα-treated cells ($*P < 0.001$). (b) In some experiments uptake was allowed to proceed to equilibrium and cells were placed in fresh medium to monitor efflux into the basolateral chamber. There was a small but significant decrease in iron efflux from the cell monolayers stimulated with TNFα ($**P < 0.01$). Data are means ± S.E.M. of 6–12 observations.

of cells with TNFα (Figs. 3(a) and 4(a)). Stimulation of cells with TNFα had no significant effect on IREG1 protein or mRNA expression (Figs. 3(b) and 4(b)). These data are supported by previous studies demonstrating that while inflammatory stimuli such as lipopolysaccharide and turpentine downregulate IREG1 mRNA in the spleen, TNFα has no effect of transporter expression [34]. Therefore, in the absence of changes in IREG1 expression in TNFα-treated cells, we conclude that the decrease in iron efflux is due to increased retention of iron within the cell possibly as a consequence of an increased rate of ferritin synthesis that could lead to its sequestration.

To gain an insight into the molecular events underlying the decrease in iron uptake across the apical membrane of the Caco-2 cell monolayer, parallel sets of cells were treated with TNFα and used to generate sources of cellular protein or total RNA. The commercially available antibodies used in this study can distinguish between the IRE and non-IRE isoforms of DMT1. Our data demonstrated that following stimulation with TNFα the protein expression of IRE-containing isoform was significantly decreased, whereas there was no effect on DMT1(non-IRE) levels (Fig. 3(c) and (d)). Data from RT-PCR analysis of RNA from TNFα-treated and control cells supported the protein studies and showed a significant decrease in DMT1(IRE) mRNA (Fig. 4(c)) but no change in DMT1(non-IRE) (Fig. 4(d)).

In total, four DMT1 splice variants have been described that exhibit sequence differences at either the 3′ [24] or 5′ end [35] of the gene. The recently described 5′ variants [35] have not been well-characterised in terms of their function and tissue abundance and therefore our study has focussed on the so-called exon 1B variants. To determine whether TNFα could alter the promoter activity of these splice variants, we cloned 1.6 kb of the 5′ flanking region upstream of the transcription start site into the pGL3 vector upstream of a luciferase reporter and transfected this construct into Caco-2 cells. Reporter gene analysis of the common DMT1 5′ promoter upstream of exon 1B showed no significant effect of TNFα treatment (Fig. 5). Previous studies using the same reporter-gene construct demonstrated that zinc (which increases DMT1 expression in Caco-2 cells [13]) strongly increases promoter activity [36], indicating that the construct is functional. The lack of effect of TNFα on promoter activity, taken together with the protein and mRNA data, suggests two possible mechanisms for the regulation of DMT1 expression. Either the exon 1A promoter plays a major role in mediating the effects of TNFα – there is some evidence that this region of the DMT1 gene is important in the overall response to iron deficiency and iron loading in

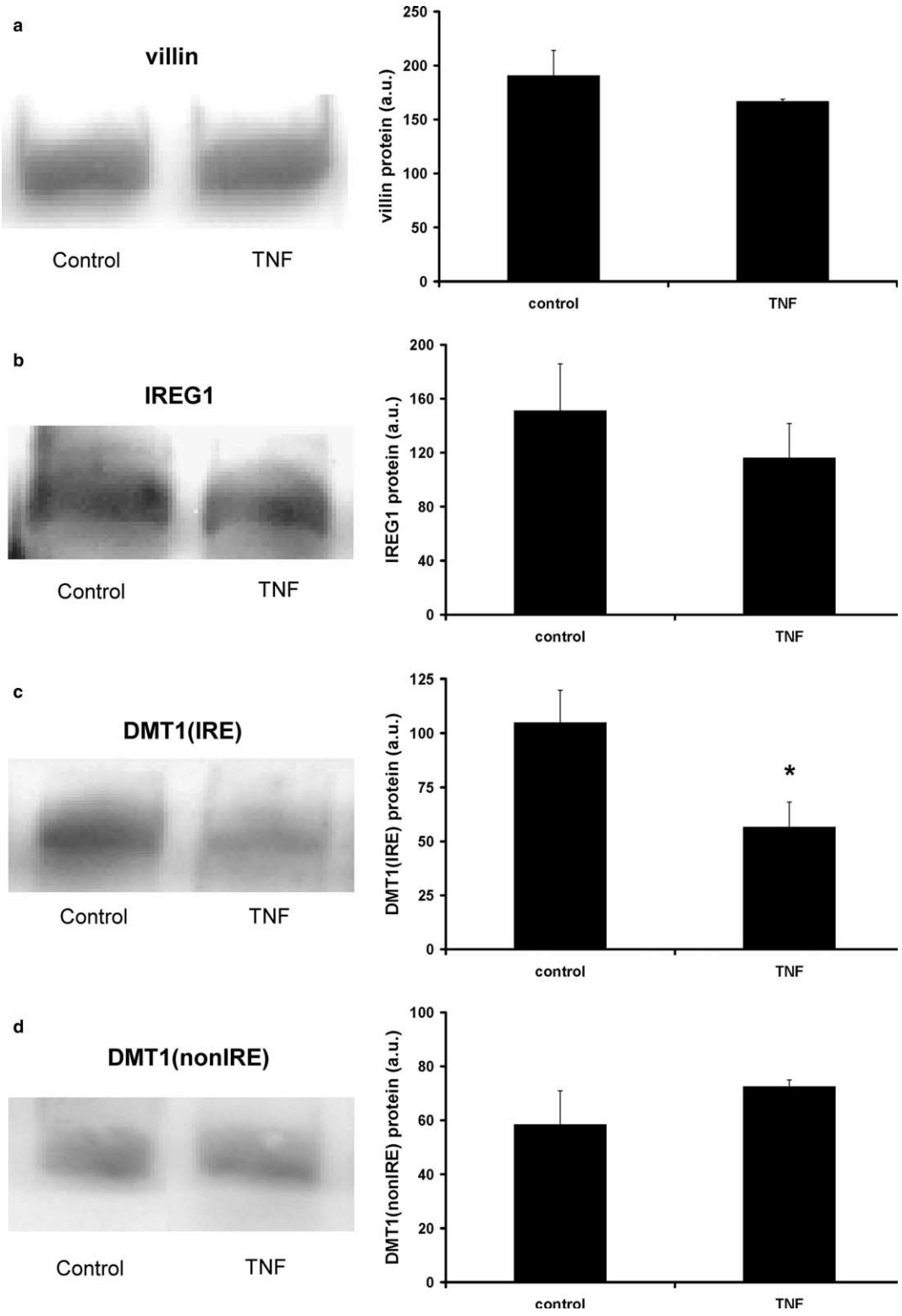

Fig. 3. Effect of TNFα on iron transporter protein expression in Caco-2 cells. Cell homogenates (20 μg protein) were resolved by SDS–PAGE, transferred to nitrocellulose membranes and probed with commercially available antibodies to (a) villin – the housekeeper protein, (b) IREG1, (c) DMT1(IRE) or (d) DMT1(non-IRE). TNFα treatment significantly decreased the expression of DMT1(IRE) (*$P < 0.03$). The expression of the other proteins was unaltered by TNFα. Densitometry data are means ± S.E.M of 3–6 separate observations.

Caco-2 cells [35]. Alternatively, the 3′ untranslated region (UTR) of DMT1 contains the regulatory information. Such a mechanism would presumably implicate a role for the IRE that resides in the 3′ UTR of one of the DMT1 splice variants [24]. In support of this possible mechanism we demonstrated that transferrin receptor (TfR) expression, which also contains IRE

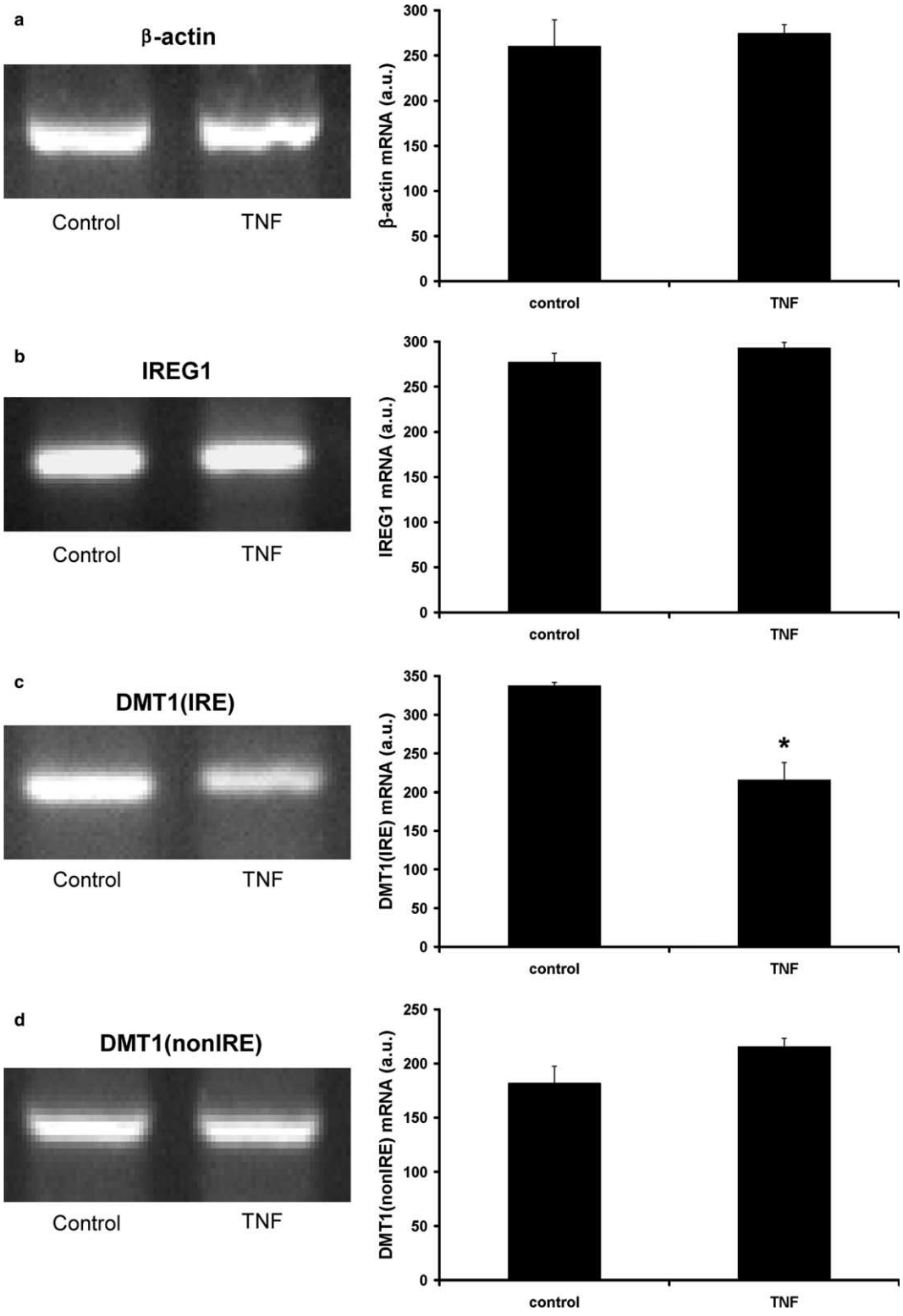

Fig. 4. Effect of TNFα on iron transporter mRNA expression in Caco-2 cells. RNA expression of (a) β-actin – control mRNA, (b) IREG1, (c) DMT1(IRE) or (d) DMT1(non-IRE) in control and TNFα-treated cells were determined by RT-PCR. Products were subjected to electrophoresis on 1% agarose gels and stained with ethidium bromide. Band intensity was determined by scanning densitometry. TNFα treatment significantly decreased the expression of DMT1(IRE) ($^*P < 0.005$). TNFα had no effect on the expression of the other transporters. Densitometry data are means ± S.E.M of six observations per group.

sequences in the 3′ UTR [37,38], was decreased in TNFα-treated cells (Fig. 6). However, while other inflammatory mediators such as lipopolysaccharide and IFNγ have been shown to decrease TfR by reducing iron regulatory protein (IRP) RNA binding activity, there is no similar evidence for a regulatory role of TNFα on IRP-1 or IRP-2 activity [39]. Thus,

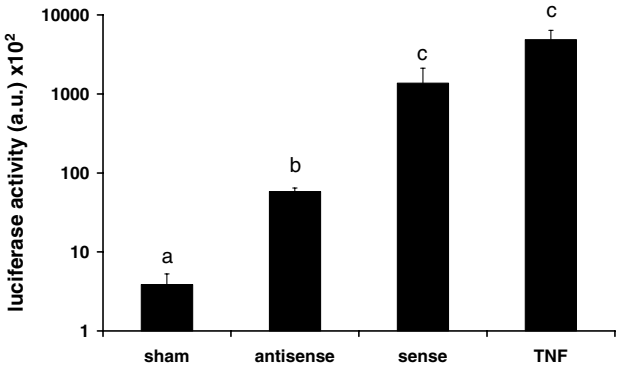

Fig. 5. DMT1 promoter activity in Caco-2 cells is not altered by stimulation with TNFα. 1.6 kb of the DMT1 5′ flanking region upstream of exon 1B was cloned into pGL3 Basic vector, creating a transcriptional fusion with a luciferase gene. Caco-2 cells were transfected with this construct and stimulated with TNFα (10 ng/ml). In some experiments, cells were subjected to sham transfection (i.e., no vector) or transfected with a vector containing the DMT1 promoter in the antisense configuration. Introduction of the sense promoter induced luciferase activity in Caco-2 cells. However, stimulation of the cells with TNFα had no further effect on luciferase activity. Data are means ± S.E.M. of 10 separate determinations in each group. Data bars containing common letter are not significantly different from each other (one-way ANOVA and Tukey's post hoc test).

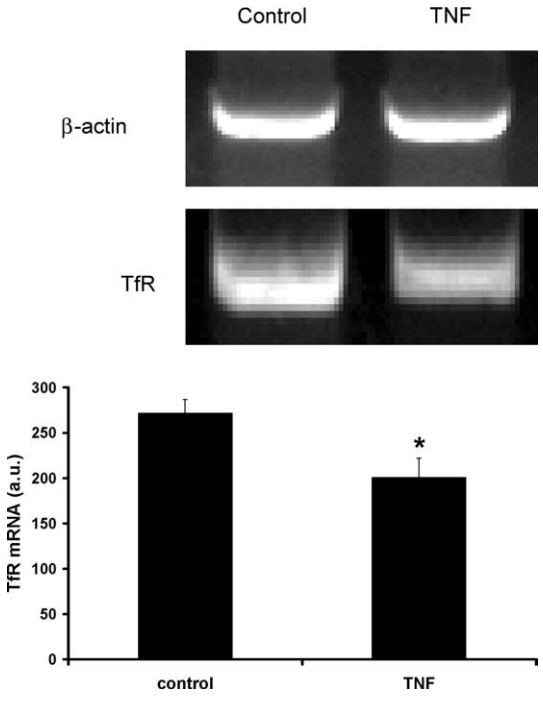

Fig. 6. Transferrin receptor expression is decreased in Caco-2 cells following stimulation with TNFα. TfR like DMT1 contains IRE sequences in the 3′ UTR. Following TNFα stimulation, TfR mRNA expression was significantly decreased in Caco-2 cells (*$P < 0.01$). There was no effect of TNFα on mRNA expression of the housekeeper gene β-actin. Densitometry data are means ± S.E.M of four experiments.

the relative roles of the 5′ and 3′ end of the DMT1 gene in mediating the response to TNFα remain to be clarified.

In summary, our data support a regulatory role for TNFα in iron metabolism. Taken together with previous studies, there remains the intriguing possibility that TNFα, in addition to its pro-inflammatory actions, could be a physiologically relevant local regulator of iron absorption following its liberation from intraepithelial lymphocytes in response to high dietary iron intake. Furthermore, the specific regulation of iron uptake across the apical membrane of Caco-2 cells together with the decrease in DMT1(IRE) expression indicates that the control of intestinal iron absorption is determined by modulation of the apical uptake machinery.

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