The Plasma Membrane Lactate Transporter MCT4, but Not MCT1, Is Up-regulated by Hypoxia through a HIF-1α-dependent Mechanism*5

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The monocarboxylate transporter MCT4 mediates lactic acid efflux from most tissues that are dependent on glycolysis for their ATP production. Here we demonstrate that expression of MCT4 mRNA and protein was increased greater than 3-fold by a 48-h exposure to 1% O2, whereas MCT1 expression was not increased. The effect was mimicked by CoCl2 (50 μM), suggesting transcriptional regulation by hypoxia-inducible factor 1α (HIF-1α). The predicted promoters for human MCT1, MCT2, and MCT4 were cloned into the pGL3 vector and shown to be active (luciferase luminescence) under basal conditions. Only the MCT4 promoter was activated greater than 2-fold by hypoxia. No response was found in cells lacking HIF-1α. Four potential hypoxia-response elements were identified, but deletion analysis implicated only two in the hypoxia response. These were just upstream from the transcription start site and also found in the mouse MCT4 promoter. Mutation of site 2 totally abolished the hypoxic response, whereas mutation of site 1 only reduced the response. Gel-shift analysis demonstrated that nuclear extracts of hypoxic but not normoxic HeLa cells contained two transcription factors that bound to DNA probes containing these hypoxia-response elements. The major shifted band was abolished by mutation of site 2, and supershift analysis confirmed that HIF-1α bound to this site. Binding of the second factor was abolished by mutation of site 1. We conclude that MCT4, like other glycolytic enzymes, is up-regulated by hypoxia through a HIF-1α-mediated mechanism. This adaptive response allows the increased lactic acid produced during hypoxia to be rapidly lost from the cell.

The transport of lactic acid across the plasma membrane is of fundamental importance for all mammalian cells. Glycolytic cells (e.g. white muscle fibers and all cells under hypoxic conditions), must rapidly export lactic acid; other tissues import lactic acid to fuel respiration (brain, heart, and red muscle) or gluconeogenesis (liver and kidney) (1–3). Transport is mediated by a family of linked monocarboxylate transporters (MCTs)2 that are also responsible for the transport of other metabolically important monocarboxylates including the ketone bodies. The MCT family has 14 members (3), 6 of which have been functionally characterized. Of these, only MCT1–MCT4 catalyze proton-coupled transport of lactate (1, 4–7). MCT1 is expressed in most cells (1, 3), whereas MCT4 is expressed strongly only in glycolytic tissues (e.g. white muscle) that must export large amounts of lactic acid (2, 8). In rats, MCT2 is found in tissues requiring high affinity uptake of lactate or pyruvate such as liver and kidney (both gluconeogenic) and neurons (lactate and pyruvate oxidation). However, MCT2 is absent in most human tissues, whereas MCT3 expression is largely restricted to the retinal pigment epithelium (see Refs. 1 and 3). MCT1, MCT3, and MCT4 require ancillary glycoproteins, either gp70 (Embigin) or, more often CD147 (Basigin), for their expression at the plasma membrane (9–10). MCT2 does not associate with CD147 (9) but requires gp70 for its functional expression (11). MCTs remain associated with basigin or embigin in the plasma membrane, and this interaction is important for their activity (11, 12).

Regulation of MCT expression by a variety of stimuli has been demonstrated, especially for MCT1 and MCT4 in skeletal muscle and heart and evidence presented for both transcriptional and post-transcriptional regulation (reviewed in Refs. 2, 3, and 13). Chronic hypobaric hypoxia has been reported to up-regulate MCT4 but not MCT1 in rat skeletal muscle (14), zebra fish gills (15), and some tumor cells (16), at least in part through a transcriptional mechanism. It is well established that increased rates of glycolysis in hypoxia are associated with greater expression of glycolytic enzymes, including the glucose transporter, Glut-1, caused by a transcriptional mechanism involving the hypoxia-inducible factor (HIF-1α) (see Ref. 17). HIF-1α is stabilized in hypoxia and up-regulates the activity of many glycolytic enzymes and other hypoxia-related genes such as those involved in angiogenesis and vasomotor regulation (see Refs. 17–19). It binds to a specific hypoxia-response element (HRE) in the promoter of hypoxia-sensitive genes where it often acts in concert with cyclic AMP response element (17, 18). Hypoxia also increases HIF-1α gene expression via a signaling pathway involving mTOR, and insulin (and other growth factors) may act synergistically to enhance HIF-1α levels through a phosphatidylinositol 3-kinase/Akt-dependent activation of the mTOR signal transduction pathway (18, 20). Because MCT4 is the predominant MCT found in glycolytic cells, it might be predicted that its expression would increase in hypoxia to enable export of the increased quantities of lactic acid. In this paper we confirm this to be the case and demonstrate that the effect is mediated by HIF-1α.

The predicted gene structures for human MCT1 (SLC16A1, chromosome 1), MCT2 (SLC16A4, chromosome 12) and MCT4 (SLC16A3, chromosome 17) are reported in the human genome databases, and for MCT1 this has been confirmed experimentally (21). A schematic representation for each is shown in Fig. 1. The intron/exon boundaries are largely conserved and there is a non-coding exon in the 5′-untranslated region, which inspection of the EST data base suggests may be spliced out in some mRNA transcripts (3). A single promoter of human MCT1

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table I and Figs. 1 and 2.
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[3] The abbreviations used are: MCT, monocarboxylate transporter; HIF, hypoxia-inducible factor; HRE, hypoxia-response element; EMSA, electrophoretic mobility shift assay; CHO, Chinese hamster ovary.
has been identified (21), whereas for chicken MCT3 the presence of two alternative promoters that are developmentally regulated has been proposed (22). However there are no published data on the MCT2 and MCT4 promoters. Here we identify the human MCT2 and MCT4 promoters and demonstrate that two hypoxia-response elements, conserved in the human and mouse MCT4 promoter, are responsible for the up-regulation of MCT4 by hypoxia. Neither MCT1 nor MCT2 are up-regulated by hypoxia because their promoters lack the corresponding HREs.

MATERIALS AND METHODS

All reagents were obtained from Sigma unless otherwise stated. HeLa and COS cells were purchased from ATCC, and wild-type and HIF-1α knock-out Chinese hamster ovary (CHO) cells were kindly donated by Peter Ratcliffe (Oxford Centre for Molecular Sciences, Oxford, UK) who also provided the spx24 promoter construct. The cardiomyocyte line HL-1 was provided by William Claycomb (LSU Health Sciences Centre, New Orleans, LA). Polyclonal antibodies against human and rat MCT1, MCT4, and CD147 were raised as described previously (11). Antibodies against cardiac-specific myosin light chain were from AbCam (Cambridge, UK), and antibodies for Glut-1 were a kind gift of Steve Baldwin (University of Leeds).

Cell Culture—HeLa and COS cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), while CHO cells were maintained in Ham’s F12 medium (Invitrogen). Both media were supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 2 mM l-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. HL-1 cells were cultured in Claycomb medium (JRH Biosciences, Lenexa, KS) as described previously (23). Cells were maintained at 37°C in 5% CO₂, 95% air at saturation humidity. For hypoxic stimulation, cultures were provided by the longest 5'-flanking region of the human MCT4 gene that was likely to contain 5'-flanking regions were amplified by PCR using primers: forward primer, Prom1F and reverse primer, Prom1R (supplemental Table 1). In all cases, the reverse primer was ProB and a KpnI site was added to the 5'-end of the forward primer and HindIII to the 3'-end of the reverse primer allowing digestion and cloning into the pGL3-basic vector, producing a 547-bp mMCT-4-luciferase construct.

Mouse MCT4 Promoter—The same strategy of 5'-mRNA and genomic sequence comparison was employed to identify 5'-flanking sequence of the mouse MCT-4 gene. An upstream region was amplified by PCR using the primers ProM4F and ProM4R shown in supplemental Table 1 and produced a 563-bp product. The presence of restriction sites KpnI to the 5'-end of the forward primer and HindIII to the 3'-end of the reverse primer allowed digestion and cloning into the pGL3-basic vector, producing a 547-bp mMCT-4-luciferase construct.

Deletion and Mutational Analysis of the MCT4 Promoter—Successive 5'-truncations of the full-length hMCT-4 promoter from −2011/+83 to −67/+83 were generated by PCR using appropriate oligonucleotide primers (supplemental Table 1). In all cases, the reverse primer was ProB and a KpnI site was added to the 5'-end of all forward primers. The resultant PCR products were subcloned into the pGL3 basic vector as described above. Site-directed mutations corresponding to the critical HIF-1 binding sites 1 and 2 (see Fig. 4) were introduced into the hMCT-4 −396/+83 construct using the QuikChange kit (Stratagen) and confirmed by sequencing (ABC Sequencing, Imperial College London, UK). The primers used are reported in supplemental Table 1.

Western Blotting of Cell Extracts—Cells were harvested, washed in phosphate-buffered saline, and pelleted at 4 °C before resuspending in ice-cold lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% (w/v) Triton X-100, 10 mM Tris-HCl, pH 7.4, containing protease inhibitors (phenylmethylsulfonyl fluoride and benzamidin at 0.5 mM) plus leupeptin, antipain, and pepstatin at 4 μg/ml). After 20 min at 0 °C,
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Quantitative PCR—Total RNA was isolated from cultured cells and DNase treated using the Ribopure RNAwiz kit (Ambion®). cDNA was synthesized from 2 μg of total RNA using 2.5 μm oligo(dT) random hexamer primers and Moleney-murine-leukemia virus reverse transcriptase (2.5 units/μl) in buffer containing 5 mM MgCl2, 1 mM dNTPs, and 4 μl of 10× Buffer, as supplied by Roche Diagnostics. Real time PCR was performed using 10 ng of reverse-transcribed total RNA with 0.8 μM sense and antisense primers (see supplemental Table 1) and 10 μl of absolute QPCR-SYBRgreen mix (ABgene), which consisted of the Thermo-start® DNA polymerase and reaction buffer in a total volume of 20 μl. The reactions were performed using DNA Engine Opticon 2 system (MJ Research, Inc.). Quantification of samples was performed using a standard curve constructed by amplifying serial dilutions of cDNA from untreated cells and plotting cycle threshold (Cv) values as a function of dilution factor from the starting concentration of cDNA.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from normal or treated cells using the nuclear extraction kit (Chemicon International). The synthetic oligonucleotides (Sigma-Genosys) used as probes or competitors were derived from the HIF-1 binding sequences present in the hMCT-4 promoter (see supplemental Table 1) and were annealed to their respective complementary strands. Labeling of the 5′-nucleotide of the double-stranded probes was performed with [γ-32P]ATP (3000 μCi/mol) using polynucleotide kinase (Roche Diagnostics). EMSAs were performed using binding reaction mixtures (20 μl) containing 10 μg of nuclear extracts with binding buffer (48 mM Hepes, 16 mM Tris, 240 mM KCl, 4 mM EDTA, 4 mM dithiothreitol, 40% (w/v) glycerol, pH 7.9) and labeled probe (20,000 dpm). After incubation at room temperature for 45 min, samples were resolved on non-denaturing 6% (w/v) polyacrylamide gel in 0.25× TBE buffer (25 mM Tris borate, 0.5 mM EDTA) at 150 V for ~3 h. The gel was dried and subjected to autoradiography. Competition and supershift experiments were performed by preincubating nuclear extract with a molar excess of relevant competitor oligonucleotide at room temperature for 15 min or 6 μg of monoclonal HIF-1α antibody (Transduction Laboratories) on ice for 30 min prior to the addition of labeled probe.

RESULTS

Hypoxia Increases Expression of MCT4 but Not MCT1—In Fig. 2A we present Western blots demonstrating that hypoxia (1% O2 for 48 h) increased the expression of MCT4 protein in HeLa, COS, and HL-1 cells whereas the expression of MCT1 was unaffected. HL-1 cells are a cardiac myocyte cell line that expresses cardiac myosin light chain whose expression, like MCT1, was also unaffected by hypoxia. In HeLa cells we confirmed the well documented up-regulation of Glut-1 by hypoxia, which is known to be mediated by HIF-1α (25). In COS cells we confirmed that the hypoxia mimic, cobalt (50 μM), also up-regulated MCT4 expression, another indicator of the role of HIF-1α (26).

In Fig. 2B we used quantitative PCR to demonstrate that the increase in MCT4 expression was accompanied by an increase in mRNA for MCT4 but not that of MCT1, which was significantly decreased by hypoxia. These data suggest that hypoxia up-regulates MCT4 expression by increasing transcription of the MCT4 gene, consistent with a role for HIF-1α.

FIGURE 1. Schematic representation of the human MCT genes. The gene structures for MCT1 (SLC16A1), MCT2 (SLC16A7), and MCT4 (SLC16A3) are those reported in the human genomic data base (www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid = 9606). SLC16A1 is found on chromosome 1 (1p12), SLC16A7 on chromosome 12 (12q13), and SLC16A3 on chromosome 17 (17q25). The predicted promoter regions were cloned from human genomic DNA (see “Materials and Methods”), and positions relative to their respective transcription start site are shown. The degree of sequence overlap between the 3′-end of the promoter constructs and the 5′-end of the first exon is as follows: hMCT1 promoter, 46 bp; hMCT2a and 2b promoters, 50 and 55 bp, respectively; hMCT4 promoter, 36 bp (the entire first exon). The genomic locations for each of the above promoters are as follows: hMCT1, 3938605–3937017 of genomic contig NT_019273.15; hMCT2a, 7255656 –7256179 and hMCT2b, 7347382–7349085, both of contig NT_086796.1; hMCT4:399042– 401136 of contig NT_010663.
The MCT4 Promoter Is Activated by Hypoxia, but the MCT1 and MCT2 Promoters Are Not—The putative human and mouse MCT promoter sequences were cloned into the pGL3 vector and promoter activity determined using the expression of firefly luciferase measured by luminescence (see “Materials and Methods”). Co-transfection with the pRL-SV40 vector encoding the Renilla luciferase gene under the control of the constitutive SV40 promoter provided a control for transfection efficiency and data are expressed as the ratio of firefly to Renilla luminescence. Data are shown in Fig. 3 Under normoxic conditions, for all promoter constructs except that for MCT2b, firefly luminescence was significantly greater than for the empty pGL3 vector promoter confirming that we had cloned the active promoter. The exception of the MCT2b promoter is not surprising because evidence for this promoter activity is only found in ESTs from testis suggesting that in other cells its activity is suppressed. The increase in both human and mouse MCT4 promoter activity by hypoxia was 2–3-fold. Spx24, a promoter containing 6 HREs was used as a positive control and gave a large (5–10-fold) increase in both human and mouse MCT4 promoter activity by hypoxia was 2–3-fold. Spx24, a promoter containing 6 HREs was used as a positive control and gave a large (5–10-fold) increase in activity under the same conditions, consistent with a role for HIF-1α.

Identification of Regions in the hMCT4 Promoter Critical for Hypoxia-inducible Transcription—Inspection of the cloned human MCT4 promoter revealed the presence of four putative HREs with the characteristic motif, 5'-RCGTG-3'. These were present at positions −1290/−1285, −511/−506, −88/−83, and −75/−70 of the promoter construct (see supplemental Fig. 1), and for each of which comprised seven replicates. The effects of hypoxia on the MCT4, mMCT4, and spx24 are statistically significant (p < 0.01).

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Their promoter activity was then tested in HeLa cells under normoxic and hypoxic conditions as shown in Fig. 5A. Truncation of the promoter to a length of 478 bp (−396/+83 construct) removed two HREs, yet the response to hypoxia was unchanged. However, further truncation to remove the final two HREs abolished any stimulatory effect of hypoxia. These data suggest that it may be the first two HREs that are responsible for the stimulation of promoter activity by hypoxia; this was confirmed by making site-directed mutants of these HRE sites as shown in Fig. 5B. When site 1 (nearest the transcription start site) was mutated there was a significant (~50%) reduction in the stimulation of promoter activity by hypoxia. However, when site 2 was mutated, stimulation by hypoxia was totally abolished as it was when both sites were mutated. Our data suggest that HRE site 2 plays the most important role in mediating the hypoxic response but that site 1 may have a facilitating role.

**HIF-1α Is Responsible for the Stimulation of MCT4 Expression by Hypoxia**—Final confirmation that it is HIF-1α that is responsible for the effect of hypoxia on MCT4 expression was provided by the use of CHO cells in which HIF-1α had been knocked out. As shown in Fig. 6, the human MCT4 promoter is active in wild-type CHO cells and shows a similar response to hypoxia to that observed in HeLa cells. Here too, the deletion of HRE sites 3 and 4 was without effect, but removal of site 2 abolished the response to hypoxia. However, in the HIF-1α knock-out cells, although basal promoter activity was normal, there was no response to hypoxia even with the full-length promoter. As expected, the response to hypoxia could be restored to the HIF-1α knock-out cells by co-transfection with HIF-1α (supplemental Fig. 2).

**Hypoxia Induces a Transcription Factor That Binds to HRE Sequences in the Human MCT4 Promoter**—Further confirmation that it is HIF-1α binding to the HRE sites that is responsible for the hypoxic response was provided by the EMSA (or gel shift) shown in Fig. 7. When a 32P-labeled DNA probe corresponding to the promoter sequence containing HRE sites 1 and 2 was mixed with nuclear extract from control and hypoxic HeLa cells, several shifted bands were observed, corresponding to the probe bound to transcription factors. Only two of these bands, the top two, were increased in the extracts from the hypoxic cells, with the greatest effect being seen on the very top band. Both bands were abolished by the presence of a molar excess of unlabeled MCT4 probe or by unlabeled probe corresponding to the HRE sites of the erythropoietin or vascular endothelial growth factor promoters (Fig. 7). These data imply that the transcription factor(s) that are up-regulated by hypoxia bind to these HRE sites.
The data of Fig. 8 confirm this in two ways. In Fig. 8A we used labeled probes in which HRE sites 1 and 2 had been mutated. Mutation of site 2 abolished binding of probe to the topmost band, whereas this was unchanged with the site 1 mutant that reduced, rather than abolished, binding to the lower band. Both effects were observed with the double mutant. In Fig. 8B we performed competition studies in which binding of labeled WT-probe was competed with excess cold probes. As predicted from the data of Fig. 8A, only probes with mutation in site 1, but not in site 2, competed strongly for the upper band. Taken together, these data are consistent with the promoter assays of Fig. 5B and suggest that it is the upper band, corresponding to the transcription factor binding to site 2, that plays the dominant role in the response to hypoxia. Binding of another factor to site 1, producing the lower band, may play a facilitatory role to enhance the activation occurring at site 2.

To demonstrate that it is HIF-1α that plays the dominant role and binds to HRE site 2, we performed the supershift assay shown in Fig. 9. This shows that incubation of nuclear extract and 3P-labeled DNA probe with an antibody against HIF-1α caused the topmost band, corresponding to the probe bound to site 2, to migrate still higher up the gel.

**DISCUSSION**

**Up-regulation of MCT4 Expression by Hypoxia Is Mediated by HIF-1α**—In this paper we have demonstrated that MCT4 expression is up-regulated in response to hypoxia through enhanced gene transcription mediated by HIF-1α. Thus we have shown an increase in both MCT4 protein and mRNA expression in response to hypoxia (Fig. 2) and demonstrated that the promoter is activated by hypoxia (Fig. 3) but not in HIF-1α knock-out cells (Fig. 6). We have identified two HRE sites just upstream of the proposed transcription start site whose mutation abolishes (site 2) or reduces (site 1) the effect of hypoxia (Fig. 5). The presence of two HREs, with an imperfect inverted repeat and separated by 8 nucleotides is a common attribute of genes regulated by hypoxia (27). As shown in Fig. 4, sites 1 and 2 of the hMCT4 promoter conform to this arrangement and the sequence is conserved in the mouse MCT4 promoter (Fig. 4B), which is also up-regulated by hypoxia (27). Gel-shift experiments have shown that site 2 binds a transcription factor that is up-regulated in response to hypoxia (Figs. 7 and 8), and confirmation that this transcription factor is HIF-1α was provided by supershift assay (Fig. 9).

Site 1 may also bind a transcription factor (Fig. 8) implying that this site plays a facilitatory role for the hypoxic response, whereas site 2 is essential (Fig. 5). The identity of this second transcription factor is unknown, but this site may fulfill the role of a HIF-1 ancillary sequence as defined by Kimura et al. (27). These authors have shown that constitutively active proteins may bind to the HIF-1 ancillary sequence site regardless of the hypoxic stimuli. However, our data demonstrate that the transcription factor binding to site 1 is enhanced by...
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hypoxia (Fig. 7), although this might be because of the increase in HIF-1α, in response to hypoxia, facilitating the further binding of a constitutively expressed protein.

The Physiological Role of MCT4 Up-regulation by Hypoxia—We have previously described how MCT4 expression is prevalent only in those tissues, such as white muscle, that rely primarily on glycolysis to provide their ATP and that the kinetic characteristics of MCT4 are especially suited for transporting lactic acid out of glycolytic cells (2, 3, 7). Thus the observed up-regulation of MCT4 in hypoxia, rather than MCT1 or MCT2, is exactly what would be predicted. Indeed, the MCT4 promoter joins those of other glycolytic enzymes that are regulated by HIF-1α, including aldolase A, enolase 1, lactate dehydrogenase A, and glucose transporter 1 (17). In vivo studies on MCT expression in hypoxia are limited, but it has been shown that in rats subject to hypobaric conditions up-regulation of MCT4 mRNA and protein is observed in slow oxidative muscles, whereas MCT1 mRNA, but not protein, is down-regulated (14). This exactly parallels what we observed in our in vitro studies on HeLa cells (Fig. 2). It is of interest that HIF-1α has been shown to be up-regulated in many cancers and is likely to play a critical role in allowing tumor cells to grow under hypoxic conditions found in solid tumors (28). Indeed, expression of MCT4 mRNA, along with other HIF-1-responsive gene products, is increased in aggressive bladder cancers consistent with their enhanced rates of glycolysis and thus their need to export large amounts of lactic acid (16). T3 increases MCT4 expression in skeletal muscle (29) and human skin fibroblasts (30), and an up-regulation of HIF-1α expression has been implicated in this effect (30).

Inspection of the MCT1 and MCT2 promoters does not reveal the presence of any likely HRE sequences, and this is confirmed by the lack of any stimulatory effect of hypoxia on their promoter activities (Fig. 3). MCT1 is up-regulated in response to increased work in both heart and muscle, although the mechanisms responsible remain to be elucidated. However, there is evidence for post-transcriptional regulation of MCT1 expression perhaps involving factors binding to the unusually long 3′-untranslated region (see Ref. 3). The expression of MCT2 is extremely species-dependent and little is known about its regulation. In the rat MCT2 expression appears to be largely restricted to those tissues, such as kidney, liver, testes, and neurons, that require a high affinity uptake mechanism for L-lactate-dependent gluconeogenesis or respiration (1, 3, 31).

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