Glucose deprivation, oxidative stress and peroxisome proliferator-activated receptor- α (PPARA) cause peroxisome proliferation in preimplantation mouse embryos

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Abstract

Ex vivo two-cell mouse embryos deprived of glucose *in vitro* can develop to blastocysts by increasing their pyruvate consumption; however, zygotes when glucose-deprived cannot adapt this metabolic profile and degenerate as morulae. Prior to their death, these glucose-deprived morulae exhibit upregulation of the H⁺-monocarboxylate co-transporter SLC16A7 and catalase, which partly co-localize in peroxisomes. SLC16A7 has been linked to redox shuttling for peroxisomal β -oxidation. Peroxisomal function is unclear during preimplantation development, but as a peroxisomal transporter in embryos, SLC16A7 may be involved and influenced by peroxisome proliferators such as peroxisome proliferator-activated receptor- α (PPARA). PCR confirmed *Ppara* mRNA expression in mouse embryos. Zygotes were cultured with or without glucose and with the PPARA-selective agonist WY14643 and the developing embryos assessed for expression of PPARA and phospho-PPARA in relation to the upregulation of SLC16A7 and catalase driven by glucose deprivation, indicative of peroxisomal proliferation. Reactive oxygen species (ROS) production and relationship to PPARA expression were also analysed. In glucose-deprived zygotes, ROS was elevated within 2 h, as were PPARA expression within 8 h and catalase and SLC16A7 after 12–24 h compared with glucose-supplied embryos. Inhibition of ROS production prevented this induction of PPARA and SLC16A7. Selective PPARA agonism with WY14643 also induced SLC16A7 and catalase expression in the presence of glucose. These data suggest that glucose-deprived cleavage stage embryos, although supplied with sufficient monocarboxylate-derived energy, undergo oxidative stress and exhibit elevated ROS, which in turn upregulates PPARA, catalase and SLC16A7 in a classical peroxisomal proliferation response.

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Introduction

Glucose deprivation is a potent cell stressor leading to the activation of cell death pathways. The totipotent cells of cleavage stage preimplantation embryos rely on monocarboxylates, pyruvate and lactate for energy during the first few divisions after fertilization (Brinster 1965, Wilding *et al.* 2002). Indeed, 5.6 mM glucose can be toxic at this time, and thus media used to culture human embryos in assisted reproductive procedures contain only very low glucose concentrations (for reviews see Biggers 1998, Lane & Gardner 2007). Gradually, co-incident with the initiation of the first differentiation into epithelial trophectoderm, glucose becomes a major ATP source. For preimplantation embryos *in vivo*, the conversion from monocarboxylate-based to glucose-based generation of ATP is induced

by prior exposure to glucose, which is provided in the oviductal milieu (Gardner *et al.* 1996, Harris *et al.* 2005), but this supply can be manipulated *in vitro*. This unusual metabolic profile presents a model for the investigation of cellular stress response induced by glucose deprivation. Subtle changes in embryo culture conditions can increase cell death and may be associated with increased reactive oxygen species (ROS) activity.

Although not used for fuel, glucose is catabolized by early cleavage stage embryos through the hexosamine biosynthetic pathway (Pantaleon *et al.* 2008) and the pentose phosphate pathway, producing amino sugar nucleotides and nucleic acid precursors that may be required for cell proliferation and NADPH generation. NADPH is predominantly used in triacylglycerol synthesis, of which there is little supporting evidence in these embryos, and in reduction of oxidized

glutathione to support glutathione peroxidase management of hydrogen peroxide (H_2O_2). So we reasoned that if stress was applied by glucose deprivation, pentose phosphate pathway flux would cease and the cells' ability to generate NADPH so as to reduce ROS would be compromised, thus potentiating further increases in cellular ROS and activation of peroxisome proliferation in order to respond.

Peroxisomes, first noted in rat oocytes *in situ* (Figueroa *et al.* 2000) and subsequently in mouse preimplantation embryos (Jansen *et al.* 2008), both produce and scavenge ROS and house enzymes for fatty acid β -oxidation (Piot *et al.* 1998) of which there is some evidence in embryos (Kane 1979, Hillman & Flynn 1980, Quinn & Whittingham 1982, Haggarty *et al.* 2006). Interestingly, SLC16A7 and catalase co-localize to embryonic peroxisomes and are upregulated in embryos deprived of glucose, which later degenerate before blastulation (Jansen *et al.* 2008). Much of this increased SLC16A7 was found in peroxisomes rather than the plasma membrane, suggesting a role in ROS metabolism. This increased peroxisomal activity suggests that glucose deprivation may result in oxidative stress activating peroxisome proliferation.

Oxidative stress arises from an elevation in ROS including superoxide anions (O_2^-) or H_2O_2 from various sources. Brief exposure of embryos to H_2O_2 inhibits cell proliferation and causes arrest before blastulation (Cebal *et al.* 2007). Similar consequences follow from early periods of glucose deprivation *in vitro* (Sakkas *et al.* 1989, Brown & Whittingham 1991, Chatot *et al.* 1994, Martin & Leese 1995). At the heart of peroxisomal proliferation is the activity of the peroxisome proliferator-activated receptors (PPAR). These ligand-activated nuclear receptors or transcription factors affect transcription of a range of peroxisome-related genes. Not surprisingly, PPARs are responsible for regulating catabolism of fats and glucose homeostasis (Lemberger *et al.* 1996b). There are three subtypes: PPARG, abundant in liver and kidney; PPARG mostly in adipose and intestinal tissue and both are activated by second messengers from the arachidonic cascade pathways. PPARG/D is constitutive to all tissues and by contrast activated preferentially by unsaturated fatty acids (Braissant *et al.* 1996, Lemberger *et al.* 1996a, Krey *et al.* 1997).

This study investigated PPARG in the metabolic stress response of early embryos because of its previously established role in fatty acid oxidation, its upregulation in response to oxidative stress (Schrader & Fahimi 2006) and after fasting (Gremlich *et al.* 2005) and in later stage ovine embryos after maternal nutrient restriction (Bispham *et al.* 2005, Budge *et al.* 2005). The fibrates WY14643, a PPARG agonist that causes peroxisomal proliferation in rodents via activation of PPARG (Nemali *et al.* 1989, Shearer & Hoekstra 2003), was used to test our hypothesis that complete glucose deprivation induced oxidative stress in the embryo with resultant induction of PPARG, SLC16A7 and catalase.

Results

Induction and phosphorylation of PPARG

Ovulated oocytes and zygotes collected at 18 h post-human chorionic gonadotrophin (hCG) express *Ppara* mRNA, which persists during culture until the two-cell stage, but could not be detected in *ex vivo* morulae (72 h post-hCG) or blastocysts (96 h post-hCG) that would develop from these zygotes (Fig. 1A). When embryos were collected and cultured, those supplied with glucose reflected this *in vivo* pattern of repression in morulae, but a weak signal persisted in glucose-deprived morulae. Studies to locate the protein were more informative. In zygotes, PPARG staining was predominantly distributed diffusely through the cytoplasm and this pattern persisted through development (Fig. 1B–I). Quantitative image analysis showed little change in PPARG over 4 h of culture, but by 8 h there was increased PPARG protein in glucose-deprived embryos, while glucose-supplied embryos showed a slight decrease in PPARG staining that persisted until 54 h post culture to the morula stage (Fig. 1G–J). Also after 8 h of glucose deprivation (Fig. 1G), staining became more granular and this more granular pattern persisted over the next 46 h (Fig. 1G–I).

Phosphorylation of PPARG was monitored with an antiserum against PPARG–phospho-Ser12 (Fig. 1K). Over the 4–24 h period, the most dramatic change was a decrease (48%, $P < 0.01$) in cellular PPARG–phospho-Ser12 staining in embryos supplied with glucose, while there was no significant change in glucose-deprived embryos. Indeed, as total PPARG levels in glucose-supplied embryos remained fairly constant through this period (Fig. 1J), the decrease in PPARG–phospho-Ser12 suggests dephosphorylation of PPARG in embryos growing under these normal conditions. In contrast, in glucose-deprived embryos, total PPARG increased 24% (Fig. 1J), while the level of PPARG–phospho-Ser12 was constant suggesting that in these embryos, although overall PPARG was induced, a smaller proportion was phosphorylated. However, the site of PPARG activity is in the nucleus, so nuclear levels of PPARG–phospho-Ser21 were examined against PPARG at 24 h with dual staining and quantitation (Fig. 1L–N). It was clear that PPARG–phospho-Ser21 concentrated about twofold in the nuclei of glucose-deprived embryos. So assuming both PPARG–phospho-Ser12 and PPARG–phospho-Ser21 behave similarly, this suggests that glucose deprivation produces cell stress leading to increased PPARG expression and greatly increased recruitment of the activated PPARG into the chromatin, possibly with contingent peroxisome proliferation. Meanwhile, embryos developing with glucose showed reduced phospho-PPARG and reduced PPARG, suggesting a quiescent metabolism (Fig. 1J and K).

Figure 1 PPARA mRNA and immunoreactivity in Quackenbush mouse embryos. (A) PCR for *Ppara* in cDNA from pools of 100 embryos per lane demonstrated an expected 412-bp band in freshly isolated oocytes and two-cell embryos as well as in two-cell embryos following *in vitro* culture for 24 h in KSOM with or without glucose (L, 1 kb ladder; O, unfertilized oocytes; Z, zygotes; 2C, two-cell embryos; M, morulae; B, blastocysts; K, mouse kidney homogenate). In embryos collected at 18 h post-hCG and cultured for 60 h, *Ppara* mRNA was detected in glucose-deprived morulae (– Glu), but not in glucose-supplied morulae (+ Glu). Embryos collected at 18 h post-hCG and cultured in KSOM were also assessed by immunofluorescence using polyclonal rabbit anti-mouse antibodies, pan-PPARA or PPARA-phospho-Ser12. Immunoreactivity for pan-PPARA is diffusely cytoplasmic at all embryo stages (B–E: 4, 8, 24 and 54 h post-culture respectively), but its intensity is reduced to absent in morulae (E) and blastocysts (not shown). Glucose-deprived embryos exhibit similar diffuse cytoplasmic staining for pan-PPARA (F–I: 4, 8, 24 and 54 h post-culture), but the intensity was increased from 8–54 h post-culture (G–I) compared with glucose-supplied embryos (C–E). Levels of PPARA were quantified and averaged to yield one value per embryo. A minimum of three embryos per treatment in each of three experiments was assayed in this way, and data were also presented for both pan-PPARA (J) and PPARA-phospho-Ser12 immunoreactivity (K). Interestingly, while total levels of PPARA-phospho-Ser12 appear to be stable in glucose-deprived embryos and decline in those cultured with glucose (K), using an antiserum against PPARA-phospho-Ser21 (red) and dual immunolabelling with pan-PPARA (green) demonstrate that nuclear PPARA-phospho-Ser21 is elevated in the glucose-deprived two-cell embryos (L and M) within 24 h of culture. ** $P < 0.01$. Quantified and presented as mean nuclear phospho-PPAR immunoreactivity from each of two nuclei in minimum of five embryos per treatment in each of three experiments.

Catalase

To investigate this possibility, the effects of the peroxisome proliferator WY14643 on transcription and translation of a classic peroxisomal marker, catalase, were examined (Fig. 2). In all embryos, cytoplasmic staining was diffuse and granular with these vesicles distributed in the cortical cytoplasm. Catalase immunoreactivity was more intense and granules were more

pronounced and numerous in glucose-deprived embryos and embryos treated with the PPAR activator WY14643 in the presence of glucose. Catalase expression was acutely sensitive to glucose deprivation or PPARA activation; as by 4 h of treatment, catalase protein was already elevated by about 20% (Fig. 2A, $P < 0.01$). Degradation of about 10% of the protein over 12 h was revealed by inhibition of translation with

Figure 2 Effect of cycloheximide (CHX) (A) and α -amanitin (C) on catalase expression in response to the presence or absence of glucose. Embryos were cultured from 18 h post-hCG, and catalase immunoreactivity was assessed and quantified in response to treatment as described using immunofluorescence. Results represent the mean $\pm$ S.E.M. grey scale intensity per embryo from a minimum of three embryos per treatment, in each of three experiments (A and C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by ANOVA and Bonferroni post-test relative to control treatments (KSOM + DMSO) in each time point. Representative images of catalase immunoreactivity following 12 h culture in response to indicated treatments are also shown. When glucose was supplied (KSOM), catalase immunoreactivity appears localized to cytoplasmic granules and sub-membranous regions and is increased in response to glucose deprivation or treatment with 10 μ M WY14643. While 2 μ g/ml cycloheximide abolished this increase (A and B), inclusion of 1 μ g/ml α -amanitin in the medium in parallel culture experiments demonstrates only partial inhibition of this stimulated increase (C and D).

cycloheximide (CHX; $P < 0.001$). Both glucose deprivation and PPARA activation increased the total pool of catalase, by increased transcription and translation ($P < 0.01$, Fig. 2A–D), but there was evidence of an inhibition of catalase turnover as even in the presence of α -amanitin, expression was increased by 50% after 12 h ($P < 0.001$).

SLC16A7

As we had already observed that SLC16A7 was located in embryonic peroxisomes, it was similarly examined to determine whether it was under PPAR regulation (Fig. 3). Both plasma membrane and granular cytoplasmic staining were 30% more intense in glucose-deprived embryos ($P < 0.001$), but SLC16A7 was not as acutely responsive as catalase (Figs 2 and 3). Studies with the protein synthesis inhibitor CHX revealed no loss of SLC16A7 over 12 h in glucose-supplied embryos (Fig. 3A and B, $P > 0.05$), but the 57% ($P < 0.001$) increase in expression induced by 12 h of glucose deprivation required both transcription and translation, suggesting transcription was activated by the glucose deprivation. The PPAR activator WY14643 produced similar increases in SLC16A7 transcription and translation to glucose deprivation, indicating that both glucose deprivation and PPARA activation induce SLC16A7 expression via transcription as well as translation.

Reactive oxygen species

The peroxisomal proliferation indicated by increased catalase and induction of PPARA induced by glucose deprivation suggested that this nutrient manipulation is a stressor, and it was hypothesized that this might be reflected in increased intracellular ROS. When these were assessed after only 2 h, ROS levels had risen by about 20% ($P < 0.05$) in glucose-deprived embryos or embryos stimulated with peroxisome proliferator (Fig. 4). Two hours later, while the ROS peak in the glucose-deprived embryos had abated, it persisted in the peroxisome proliferator-treated embryos, indicating a more persistent induction. To confirm a role for peroxide production in this ROS peak, the experiment was repeated using diphenyleneiodonium (DPI) to inhibit flavoenzymes, particularly NADPH oxidase, and so decelerate the ROS synthesis resulting from glucose deprivation. Again, in the glucose-deprived and PPARA agonist-treated zygotes, ROS levels were about 20% greater than for zygotes developing with glucose (KSOM), but inhibition of flavoenzymes with DPI prevented or reduced this elevated ROS arising from glucose deprivation or PPARA agonism (Fig. 5).

Effect of DPI on PPARA and SLC16A7 expression

This suggested that the induction of PPARA might be downstream from the increased ROS. So some embryos from each group were left in culture for 8 or 24 h, before

Figure 3 Effect of cycloheximide (A) and α -amanitin (C) on SLC16A7 expression in response to the presence or absence of glucose. Embryos were cultured from 18 h post-hCG and SLC16A7 immunoreactive expression was assessed and quantified as described. Results represent the mean $\pm$ s.e.m. grey scale intensity per embryo from a minimum of three embryos per treatment, in each of three experiments (A and C); *** $P < 0.001$ by ANOVA and Bonferroni post-test relative to control treatment within each time point. Representative images of SLC16A7 immunoreactivity following 12 h of culture in response to indicated treatments are also shown. When glucose was supplied (KSOM), SLC16A7 immunoreactivity, which appears localized to cytoplasmic granules as well as on the plasma membrane, is elevated both in response to glucose deprivation and treatment with 10 μ M PPARA agonist WY14643. While treatment of embryos with both 2 mg/ml cycloheximide and 1 μ g/ml α -amanitin had no apparent effect at 4 h of culture, both treatments completely inhibit this stimulated increase in SLC16A7 expression following 12 h of culture (C and D).

they were fixed and subsequently assessed by immunofluorescence for either PPARA (at 8 h, Fig. 6) or SLC16A7 (24 h, Fig. 7). Mean grey levels in zygotes cultured in KSOM (Figs 6A and 7A and white bars in corresponding graphs) were accepted as control levels, and all other mean grey levels were compared with controls. In glucose-deprived zygotes (Fig. 6B and graph), PPARA immunoreactivity was about 15% more intense ($P < 0.05$), DPI completely blocked this increase (Fig. 6C), but was ineffective on the quantitatively similar WY14643 induction of PPARA (Fig. 6D and E and graph). This pattern was replicated by SLC16A7, except that the glucose deprivation (Fig. 7B and graph) or WY14643 induction (Fig. 7D and graph) was almost 150% in both cases, possibly reflecting the different times of observation, 8 h for PPARA and 24 h for SLC16A7.

Discussion

Although reliant upon monocarboxylates rather than glucose for ATP production, cleavage stage mammalian embryos are exquisitely tuned to glucose supply. Given that glucose deprivation is a potent cell stressor, that glucose can act as a signalling molecule to permit metabolic differentiation (Pantaleone *et al.* 2008) and that glucose deprivation increases partly peroxisomal

SLC16A7 expression (Jansen *et al.* 2008), we set out to examine the impact of glucose deprivation on early embryonic oxidative stress.

PPARA, SLC16A7 and catalase

PPARA is a nuclear receptor from the steroid receptor superfamily (Issemann & Green 1990) activated by concurrent dimerization with RXR, phosphorylation of specific sites (including Ser12 and Ser21) and the attachment of activating ligands, including unsaturated fatty acids and derivatives of the lipooxygenase route of the arachidonic acid cascade (Diradourian *et al.* 2005). Activation of PPARA tends to induce further expression of PPARA (Sharifpanah *et al.* 2008) and subsequent peroxisome proliferation, including the transcription of peroxisomal β -oxidation-encoding genes such as acyl-CoA oxidase, multifunctional protein and thiolase (Bardot *et al.* 1995).

Never examined in preimplantation embryos, PPARA has been detected in mouse embryonic stem (ES) cells (Sharifpanah *et al.* 2008) that originate as pluripotent cells of the inner cell mass (ICM) of the day 4 embryo or blastocyst where they are completely isolated from the external/maternal milieu by the epithelial trophectoderm. ICM cells convert all glucose supplied via the trophectoderm to lactate (Hewitson & Leese 1993).

Figure 4 ROS production in mouse embryos deprived of glucose. Mouse embryos (CBA/F1) were collected at 18 h post-hCG, cultured for 2 or 4 h in standard KSOM, KSOM-lacking glucose (KSOM-G) or KSOM with 10 μ M WY14643 and ROS assayed as described in Materials and Methods. Data represent mean fluorescence of culture groups divided by mean fluorescence of average esterase activity from randomly sampled embryos. Within 2 h of culture, ROS levels in glucose-deprived embryos or WY14643-treated embryos are higher than those in embryos cultured in standard KSOM (* P <0.05). While the ROS levels remain high in the presence of WY14643 at 4 h post-culture (* P <0.05), in glucose-deprived embryos they decline to reach levels not significantly different from glucose-supplied embryos (P >0.05).

Ppara mRNA is expressed in the zygote and two-cell mouse embryo, but cannot be detected in pooled cDNA from the equivalent of four morulae cultured with glucose. Similarly, the protein disappears from glucose-supplied embryos as they develop through the cleavage stages *in vitro*. But if embryos are glucose deprived, the mRNA persists in morulae and the protein is upregulated after 8 h deprivation. Moreover, glucose deprivation prevented the dephosphorylation of PPARG-Ser12 that occurs when glucose is supplied. More importantly, it doubled the concentration of nuclear phospho-PPARA at 24 h, consistent with increased transcriptional activity associated with, among other things, peroxisome proliferation. This pattern was replicated when glucose-supplied embryos were treated with the PPARG agonist WY14643. So one mechanism triggered by glucose deprivation involves nuclear recruitment of phospho-PPARA with consequent peroxisomal proliferation and likely metabolic re-orientation. In different cells and with different stimuli, phosphorylation can activate or deactivate PPARG (Gelman *et al.* 2005). Supporting these results, there is evidence that glucose deprivation stabilizes *PPARG* mRNA, reducing its degradation, and increases mRNA levels for PPARGC1A, a major co-activator of PPARG (Bogdanova *et al.* 2007). Several groups have reported upregulation of PPARG activity in response to fasting (Sterchele *et al.* 1996, Hashimoto *et al.* 2000, Escher *et al.* 2001, Sugden *et al.* 2001) and in sheep, maternal nutrient restriction between 28 and 80 days gestation induced PPARG expression in offspring (Bispham *et al.* 2005, Budge *et al.* 2005). In addition to this induction and activation by glucose deprivation, PPARG activation rapidly increased PPARG levels by parallel self-induction of transcription and also through inhibited

polyubiquitination and proteasomal degradation (Hirotani *et al.* 2001).

Results herein demonstrate that PPARG activation induced SLC16A7 expression even when glucose was supplied, because transcriptional and translational inhibitors had the same degree of inhibition of induction. This suggests that activated PPARG, possibly phospho-PPARG, acts to promote SLC16A7 expression. SLC16A7 has not been identified as a target protein for peroxisome proliferation, but PPARGs affect target genes by binding to a specific DR1 motif in a peroxisome proliferator-response element (PPRE) located upstream in the promoter region (Latruffe *et al.* 2000). A brief assessment of the *Slc16a7* gene identified such a DR1 sequence as well as sites for many stress-associated transcription factors such as: GATA; CREB; FKHD; HIF; and NFkB (Table 1), providing further support for the conclusion that the PPARG activation is upstream of SLC16A7 induction. Because of the association of MCT with peroxisomes and peroxisomal metabolism, it is likely that SLC16A7 is induced along with other key peroxisomal proteins during peroxisomal proliferation.

Catalase, on the other hand, appeared to have different regulatory modes from SLC16A7. Both glucose deprivation and PPARG agonism (WY14643) increased catalase expression even after 4 h of culture. These results are supported by evidence of PPARG induction upregulating catalase (Nemali *et al.* 1989) and the existence of a functional PPRE in the catalase promoter (Girnun *et al.* 2002). As in mouse zygotes, in both these studies, α -amanitin treatment only marginally reduced the increase in catalase immunoreactivity, suggesting

Figure 5 Effect of DPI on embryonic ROS production. CBA/F1 mouse zygotes were collected at 18 h post-hCG, cultured for 2 h in standard KSOM, KSOM-lacking glucose, KSOM-lacking glucose with 0.1 μ M DPI, KSOM with 10 μ M WY14643 in DMSO, KSOM with 10 μ M WY14643 in DMSO and 0.1 μ M DPI, KSOM with DMSO 1:10 000. They were then assayed for ROS as described in Materials and Methods. Data represent mean fluorescence of culture groups divided by mean fluorescence of average esterase activity from randomly sampled embryos. The addition of DPI into the media of glucose-deprived embryos reduced ROS levels in these embryos to a level that not statistically different from that in glucose-supplied embryos. In embryos cultured with WY14643, DPI reduced ROS levels, but they remained significantly higher than those in glucose-supplied embryos. Means with different superscripts are significantly different (P <0.05). Results from embryos cultured in KSOM+DMSO 1:10 000 were not statistically different from KSOM alone (not shown).

Figure 6 Effect of DPI on embryonic PPARA. Three experimental sets of CBA/F1 mouse embryos cultured for 8 h under varied conditions were fixed and underwent simultaneous immunocytochemistry for PPARA. Immunoreactivity for PPARA in all embryos was diffused throughout the cytoplasm and stained weakly in nuclei, but was noticeably absent from nucleoli. Representative embryos from groups cultured in: KSOM (A, glucose supplied), KSOM-lacking glucose (B, glucose deprived), KSOM-lacking glucose with 0.1 μ M DPI (C), KSOM with 10 μ M WY14643 in DMSO (D), KSOM with 10 μ M WY14643 in DMSO and 0.1 μ M DPI (E) are shown. The average grey levels for glucose-deprived embryos or treated with WY14643 were higher than for glucose-supplied embryos. When DPI was added to glucose-deprived embryos, the pan-PPARA staining was reduced to levels not statistically different from that of glucose-supplied embryos. This was not the case for embryos cultured with WY14643 and DPI, where pan-PPARA staining remained elevated. Means with different superscripts are significantly different ($P < 0.05$).

a more complex regulation of catalase involving transcriptional and post-transcriptional levels. This evidence has been confirmed in many investigations that have identified post-transcriptional controls for catalase activity (Yano *et al.* 1998, 2004, Yano & Yano 2002, Cao *et al.* 2003, Vivancos *et al.* 2004). There is evidence that proteasomal degradation of catalase in response to stress is increased in a biphasic pattern via a pathway involving activation of the ABL1 non-receptor tyrosine kinase and the product of the *ABL1*-related gene (*Arg*). It may be that the level of H_2O_2 generated in the glucose-deprived embryos was insufficient to promote binding of ABL1 and *Arg* to catalase thus increasing stability (Cao *et al.* 2003). Additionally, because embryonic genome activation does not occur until the mid-late two-cell stage, it is possible that a greater part of catalase induction relies on pre-formed maternal mRNA delivered in the oocyte and not yet degraded.

Glucose deprivation by preventing pentose phosphate pathway flux and NADPH production induces ROS leading to PPARA induction and peroxisome proliferation. However, PPARA activation similarly leads to ROS and peroxisome proliferation. Investigation herein of the source of ROS in embryos using the NADPH oxidase inhibitor, DPI, revealed attenuation of the ROS peak

generated by PPARA agonists, but not completely. In contrast, in mouse ES cells, WY14643-induced activation of PPARA caused ROS elevation that was attenuated with DPI, but not the mitochondrial electron transport inhibitor rotenone (Sharifpanah *et al.* 2008). However, it is possible that the extended ROS peak produced by the PPARA agonist might arise from the contribution of ROS arising from sources other than those involved in glucose deprivation-induced ROS. It has been demonstrated that hepatocytes in NADPH oxidase-deficient (*p47^{phox}*-null) mice treated with WY14643 still exhibit acute increased ROS with a rapid increase in oxidative DNA damage and increased cell proliferation, a response that is potentially mediated through nuclear factor kappa B (Woods *et al.* 2007). These observations may explain why the DPI protocol was ineffective in reducing ROS levels in agonist-treated embryos to the same extent as those deprived of glucose. Alternatively, the extended ROS peak may arise from β -oxidation of lipids (Piot *et al.* 1998, Teissier *et al.* 2004, Poirier *et al.* 2006). The 10 μ M WY14643 used in these experiments is likely to be far in excess of the concentration of activated PPARA induced by glucose deprivation, and it is not unreasonable to assume that exaggerated oxidative stress is occurring in this situation. Studies in rat liver have shown that PPARA induction by

Figure 7 Effect of DPI on embryonic SLC16A7. Three experimental sets of CBA/F1 mouse embryos cultured for 24 h under varied conditions were fixed and underwent simultaneous immunocytochemistry for SLC16A7. In all embryos, SLC16A7 staining was located on the plasma membrane and in the cytoplasm as stippling or tiny granules. Representative embryos from groups cultured in: KSOM (A, glucose supplied), KSOM-lacking glucose (B, glucose deprived), KSOM-lacking glucose with 0.1 μ M DPI (C), KSOM with 10 μ M WY14643 in DMSO (D), KSOM with 10 μ M WY14643 in DMSO and 0.1 μ M DPI (E) are shown. When quantified, SLC16A7 staining in glucose-deprived embryos or in glucose-supplied embryos treated with WY14643 was higher than for embryos cultured in KSOM alone. When DPI was added to glucose-deprived embryos, SLC16A7 staining was reduced to levels not statistically different from those of glucose-supplied embryos. This was not the case for embryos treated with WY14643 and DPI whose SLC16A7 staining remained significantly elevated. Means with different superscripts are significantly different ($P < 0.05$).

Table 1 Putative transcription regulatory sequences in the 2.4 kb region upstream of the mouse *Slc16a7* gene.

Matrix name	Position ^a	Strand ($\pm$) ^b	Core similarity	Matrix similarity	Sequence (capitals: core sequence)
PPAR/RXR DR1 site	31–53	(–)	1.000	0.950	gtttctcaGGAAGagagagca
GATA	197–209	(+)	1.000	0.968	tccaGATAatctg
	516–528	(–)	1.000	0.963	tggaGATAaagga
	1181–1193	(–)	1.000	0.970	tccaGATAggtag
	1806–1818	(+)	1.000	0.976	aacAGATctaacc
	2158–2170	(–)	1.000	0.916	ggaAGATtataaa
	2382–2394	(+)	1.000	0.934	gggAGATcataaa
CREB	247–267	(–)	1.000	0.990	ttgtgtGACGtgcaatttc
	379–399	(–)	1.000	0.900	cacagcTGATgtatgagct
	1186–1206	(–)	1.000	0.941	acaataTGACgtttccagata
	2287–2307	(–)	1.000	0.905	acttcaTGACttaatatcaaa
FKHD	250–266	(+)	1.000	0.834	tgacagtcAACAcaaag
	1808–1824	(+)	1.000	0.904	cagatcTAAAcaggacg
	2361–2377	(+)	1.000	0.787	tctacgaaAACActgag
HIF	247–263	(–)	1.000	0.942	tggttgacCGTGcaata
	2091–2107	(–)	1.000	0.923	gttttctaCGTGacca
NFKB	360–372	(–)	1.000	0.996	ttgggggtTTCct
	651–663	(+)	1.000	0.937	gcGGGAggtccca
	652–664	(–)	1.000	0.938	atGGGAcctcccg

The 2.4 kb sequence upstream of the *Slc16a7* ATG of the mouse sequence (GenBank: NM_011391) was analysed by MatInspector, a transcription factor database.

^aThe positions of the putative binding sites are shown as the distance (bp) from the first nucleotide of the mouse *Slc16a7* promoter region (GenBank: NM_011391). ^bThe (+) and (–) indicate that the putative motifs are located on the plus or minus strands of this upstream 2.4 kb sequences.

peroxisome proliferators produces differential regulation of peroxisomal enzymes (Nemali *et al.* 1989, Yeldandi *et al.* 2000). WY14643, in particular, induced a 20-fold increase in the genes encoding peroxide-generating enzymes like fatty acyl-CoA oxidase and cytochrome P450 (CYP) 4A isoforms for peroxisomal β -oxidation within 30 min to 1 h, while catalase displayed only a 2-fold increase. This results in overwhelming generation of H_2O_2 within peroxisomes, with possible overflow into the cytoplasm.

While there is very little information on peroxisomal activity in embryos, it is pertinent to note that a knock-out model for the mitochondrial fatty acid oxidation enzyme long-chain acyl-CoA dehydrogenase (LCAD) appears to block mouse embryo development at the morula stage (Berger & Wood 2004). This group demonstrated that *Lcad*^{–/–} embryos cannot complete the development of a blastocoele and formation of a blastocyst, and subsequently degenerate at the morula stage, similarly to embryos deprived of glucose. Supplementation with shorter chain fatty acids that could be readily oxidized did not rescue development, suggesting that longer chain fatty acid oxidation does occur and plays a vital role in mouse development. LCAD and many other mitochondrial β -oxidation enzymes (L-type carnitine palmitoyltransferase I and medium-chain acyl-CoA dehydrogenase) are regulated by PPARA (Ringseis *et al.* 2007). This brings an interesting insight into our experiments. It seems that while PPARA appears to be activated in the presence of glucose, this in itself is not enough to overcome the block to development. Perhaps this is because mouse embryos at this early stage do not have enough fatty acid stores to

derive enough energy via β -oxidation. The addition of exogenous medium and long-chain fatty acid substrates in the absence of glucose may have allowed these embryos to progress. This question needs further study.

The importance of fatty acid oxidation to early development is further illustrated by the benefits to blastulation of supplementation with long-chain fatty acids in rabbit (Kane 1979) and mouse (Quinn & Whittingham 1982); while in mice, palmitic acid oxidation, constant until the eight-cell stage, accelerates as blastulation progresses (Hillman & Flynn 1980), providing a metabolic explanation for this benefit. In humans, palmitic acid uptake increases to the morula stage before declining co-incidentally with increased linoleic acid uptake and blastulation (Haggarty *et al.* 2006). Supporting evidence for preimplantation peroxisomal function now exists in the present association of SLC16A7 with oxidative stress induced by glucose deprivation or PPARA activation. SLC16A7 might exist in the peroxisomal membrane to facilitate pyruvate import for conversion to lactate via peroxisomal LDH. As hypothesized by McClelland *et al.* (2003) in relation to rat liver peroxisomes, this would enable recycling of NAD^+ for continued peroxisomal β -oxidation of long-chain fatty acids and lactate export to the cytoplasm could be facilitated by SLC16A7, either for subsequent mitochondrial metabolism or facilitated excretion to the oviduct. Adding further support to this argument, providing high lactate concentrations in the incubation medium reduced not only pyruvate utilization, but also the $NAD^+/NADH$ ratio (Lane & Gardner 2000). So it is possible that preimplantation embryos do utilize their peroxisomes for lipid oxidation and that peroxisomal

proliferation leads to increased ROS production from this source.

The association of PPARA activation and cardiomyogenic inducing transcription factors (Sharifpanah *et al.* 2008) perhaps reflects metabolic differentiation to lipid oxidation dependent rather than glycolytic, metabolic profile. This is not too different from the requirements of the glucose-deprived cleavage stage embryo that must rely on accelerated uptake of monocarboxylates or perhaps exogenous oocyte-derived lipid. There is evidence in species where oocytes are more lipid loaded than mice, of lipid metabolism being related to developmental potential (Zeron *et al.* 2001, Boni *et al.* 2002, Nagano *et al.* 2006). The analogy between the embryo and ES cells is reinforced by the observation that PPARA activation leads to increased intracellular ROS. In both cases, NADPH oxidase inhibited by DPI contributes to this ROS, but while this is the only ROS source induced in ES cells in embryos, because DPI did not limit ROS concentrations to unstimulated levels, it is likely that other sources also contribute, such as lipid oxidation or mitochondrial respiratory chain. There is a parallelism in the metabolic fate directed by PPARA activation in ES cells and early embryos. ES cells are derived from ICM cells that are known to rely on oxidation of glucose to lactate for energy (Lonergan *et al.* 2007). The early embryonic cells generate their ATP from of monocarboxylate oxidation. Adult cardiomyocytes oxidize long-chain fatty acids; fetal heart prefers glucose/lactate (van der Vusse *et al.* 1992). Metabolic differentiation from the progenitor cells thus must produce increased peroxisomal and associated MCT activity. As this study demonstrates, this is exactly the outcome of fibrate activation of PPARA in totipotent embryonic cells.

The unusual metabolic profile of the early embryo, relying on mitochondrial ATP generation from the monocarboxylic acids such as pyruvate and lactate, has proved a useful model. The small amounts of glucose normally taken up are thought to be metabolized by the pentose phosphate pathway for the maintenance of reducing power in the cell and in nucleic acid and lipid biosynthesis. In this study, glucose deprivation ablates pentose phosphate pathway activity, of itself limiting NADPH production, but also prevents the dynamic rerouting of metabolic flux to the pentose phosphate pathway, which has been proposed as a conserved post-translational response to oxidative stress (Ralser *et al.* 2007). This suggests that the use of glucose-deficient media in modern-assisted reproductive technologies might require some experimental re-examination.

We have demonstrated that very early embryonic cells experience glucose deprivation as an acute stressor, despite their novel metabolic reliance on monocarboxylates. Within only 2 h of glucose deprivation, ROS was elevated, increasing PPARA levels that preceded peroxisomal proliferation. PPARA activation also leads to peroxisomal proliferation and increased ROS.

Interestingly, although not required for energy metabolism, provision of glucose as would be normal *in situ* does not support induced ROS or peroxisome proliferation, suggesting an important stress monitor role for glucose.

Materials and Methods

Ethics

The Animal Ethics and Experimentation Committees of the University of Queensland and the University of Adelaide approved all experiments on mice. These committees are approved by the National Health and Medical Research Council of Australia.

Media, chemicals and buffers

Handling media

M2 medium (Fulton & Whittingham 1978) modified to contain 0.33 mM sodium pyruvate and 4 g/l of BSA (Sigma-Aldrich, fraction V, A-9647) and lacking glucose.

Culture media

KSOM containing 0.2 mM glucose but no amino acids (Erbach *et al.* 1994). WY14643 (Sigma-Aldrich), a selective PPARA agonist and peroxisome proliferator, was tested for toxicity on zygotes 18–20 h post-hCG at concentrations of 0.1–10 μ M in KSOM. None of these concentrations affected the proportion of embryos developing to the blastocyst stage by 96 h post-hCG, so 10 μ M were selected for experiments. CHX (2 mg/l CHX, Sigma-Aldrich (Heo & Han 2006)), and α -amanitin (1 mg/l α AA, Fluka, 06422 from Sigma-Aldrich (Liu *et al.* 2004)), were used to inhibit translation and transcription respectively. DPI (Sigma-Aldrich), an irreversible inhibitor of flavoenzymes, particularly activated NADPH oxidase, with tenfold lower potency for NADH oxidase (Morre 2002), inhibits the production of peroxide by the blockade of several NADP-dependent enzymes in the pentose phosphate pathway and tricarboxylic acid cycle. DPI was toxicity tested on zygotes 18–20 h post-hCG at concentrations of 0.01–10 μ M in KSOM, for 20 and 40 min exposure times and 20 min exposure to 0.1 μ M. DPI was used in experiments because it did not affect blastocyst development and was the EC50 for HeLa cell NADPH oxidase (Morre 2002).

Homogenization buffer

Fifty millimoles Tris-HCl; 150 mM NaCl; 10 mM NaF; 1 mM Na_3VO_4 ; 1% Triton X-100; 1 complete protease inhibitor tablet per 50 ml buffer (Roche Diagnostics Australia P/L).

Laemmli sample buffer (Laemmli 1970)

Ten percent SDS containing 1% glycerol; 0.125 g/l bromophenol blue; 0.125 g/l xylene cyanol; 100 mM dithiothreitol; 125 mM Tris-HCl, pH 6.8. Towbin transfer buffer (Towbin *et al.* 1979): 25 mM Tris; 192 mM glycine; 20% methanol, pH 8.3.

Embryo collection and culture

For some experiments (Figs 1–3), female 6–8-week-old Quackenbush mice from a high fecundity colony at the University of Queensland were injected with 10 IU equine chorionic gonadotrophin (eCG, Folligon, Intervet P/L, East Bendigo, VIC, Australia) and 48 h later with 10 IU hCG (Chorulon, Intervet). In experiments involving H₂O₂ (Figs 4–7), CBA/F1 hybrid mice from a colony at the University of Adelaide were injected with 5 IU eCG (Sigma–Aldrich) and 48 h later with 5 IU hCG (Pregnyl; Organon Inc., Roseland, NJ, USA). Embryos were collected *ex vivo* at 24, 48, 72 and 96 h post-hCG into M2 medium (Fulton & Whittingham 1978), modified to contain 0.33 mM sodium pyruvate and 4 g/l of BSA and lacking glucose. Cumulus cells were removed where necessary using 0.5 g/l hyaluronidase (Sigma–Aldrich) in glucose-free M2, then washed thrice in fresh glucose-free M2.

For experiments *in vitro*, zygotes (18 h post-hCG) were collected into glucose-free M2 and cultured in KSOM (Erbach *et al.* 1994) microdroplets under mineral oil at a density of 1 embryo/ μ l in 4.7% O₂, 4.7% CO₂, 6.1% H₂O and 84.5% N₂ at 37 °C in a MINC incubator (Cook, Eight Mile Plains, QLD, Australia). KSOM contained 0 mM (KSOM-G, glucose deprived) or 0.2 mM glucose (KSOM, glucose supplied), and in some experiments variations of both KSOM and KSOM-G contained 10 μ M WY14643 or 0.1 μ M DPI or both. Cultured embryos were sampled at various stages for different experiments as described.

mRNA analysis

Total RNA was extracted from pools of 100 morphologically normal embryos collected after 30 or 54 h culture of zygotes collected at 18 h post-hCG, using phenol/chloroform and the RNeasy Mini Kit (Qiagen). For positive controls, RNA was extracted from mouse kidney or liver and 1 μ g used for RT. RNA was reverse transcribed and amplified by PCR (Arcellana-Panlilio & Schultz 1993) using specific primers for *Ppara*: 5' primer, 5'-tgatgagatgcgctctct-3'; 3' primer, 5'-ggtagaagactgcagtag-3'. Agarose gel (1%) containing 0.5 mg/l ethidium bromide was used to resolve the 412-bp product. All cDNA samples were tested for genomic contamination using parallel PCR for mouse β -actin with primers: 5' primer 5'-cgtggccgccctaggcacca-3'; 3' primer, 5'-ttggccttaggttcagggggg-3', which span the first intron of the mouse β -actin gene producing a 243-bp cDNA fragment and a 330-bp fragment from contaminating genomic DNA (Telford *et al.* 1990). Using Gel Extraction Kit (Qiagen), PCR products were extracted and ligated into a pGem-T Easy Vector (Promega), then amplified in transformed DH5 α *Escherichia coli* cells and purified using QIAprep Spin Miniprep Kit (Qiagen). EcoRI (Roche Diagnostics GmbH) was used to digest purified plasmid clones, which were then sequenced by the Australian Genome Research Facility (Brisbane, QLD, Australia) to confirm sequence identity.

Antisera

Affinity-purified anti-SLC16A7 IgG was raised in goats against a synthetic peptide mapping to the carboxy terminus of mouse

SLC16A7 (14926, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-catalase antiserum was raised in rabbits against whole purified bovine liver catalase (200-4151, Rockland Immunochemicals Inc., Gilbertsville, PA, USA). Affinity-purified anti-PPARA IgG was raised in rabbits against a synthetic peptide corresponding to N-terminal amino acids 1–18 of mouse PPARA (ab8934, Abcam, Cambridge, UK), amino acids 8–19 of serine-12-phosphorylated mouse PPARA (Abcam ab3484) and amino acids 14–25 of serine 21-phosphorylated mouse PPARA (Abcam ab3485). Secondary antibodies used for immunofluorescence or western immunoblotting included: FITC-conjugated rabbit anti-goat IgG (Calbiochem, EMD Chemicals Inc., San Diego, CA, USA); FITC-conjugated goat anti-rabbit IgG (Calbiochem); Texas Red-conjugated goat anti-rabbit IgG (Calbiochem); HRP-labelled donkey anti-rabbit IgG (Amersham Biosciences); or rabbit anti-goat IgG (Progen Biosciences, Toowoong, QLD Australia).

Immunofluorescence

Embryos fixed in 2% paraformaldehyde in PBS (pH 7.4) underwent immunohistochemical processing (Pantaleon *et al.* 1997). Briefly, overnight incubation in primary antibody (2–10 mg/l) at 4 °C was followed by exposure to secondary antiserum for 1 h at room temperature. Samples were mounted in glycerol on concave glass slides and observed with a Bio-Rad MRC-1024 confocal laser-scanning microscope mounted on a Zeiss (Oberkochen, Germany) Axioskop using a Zeiss Plan-APOCHROMAT $\times 60$ oil immersion objective. Where necessary for statistical analysis, after fixing, triplicate experimental sets were accumulated and held for 1–3 days at 4 °C reducing variability.

Western immunoblotting

Mouse liver was homogenized in ice-cold homogenization buffer and centrifuged at 14 000 *g* for 15 min at 4 °C. Supernatant was removed and mixed with an equal volume of Laemmli sample buffer. Pools of 100 embryos in minimal medium were placed in 5 μ l homogenization buffer with equal volume of Laemmli sample buffer. Liver and embryo samples were separated by PAGE and transferred via semi-dry electrotransfer to 0.45 μ m Immobilon-P PVDF membrane (Millipore P/L, North Ryde, NSW, Australia) using Towbin transfer buffer. Membranes were incubated at room temperature for 1 h in blocking solution of 7.5% BSA in PBS with 0.1% Tween-20, before incubation with primary antiserum (0.1 mg/l) overnight at 4 °C. Finally, membranes were exposed for 1 h at room temperature to donkey anti-rabbit secondary antibody conjugated to HRP, and bands revealed with Supersignal West Femto Enhanced Chemiluminescence Detection Kit (Pierce Biotechnology Inc., Rockford, IL, USA) on X-ray film developed in a Kodak M35 X-Omat Processor (Kodak Australasia P/L).

ROS assay

Levels of ROS generation in embryos were assessed fluorometrically using 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA, Sigma–Aldrich) as previously described (Nasr-Esfahani *et al.* 1990). Esterases within the cells cleave

the acetate groups trapping the reduced form of the probe (DCHF) intracellularly, which is readily oxidized to dichloro-fluorescein (DCF) by H_2O_2 or $\text{OH}^\cdot$, but is relatively insensitive to (Vanden Hoek *et al.* 1997, Hempel *et al.* 1999). Zygotes (18–20 h post-hCG) from CBA/F1 hybrid mice collected into M2-lacking glucose were stripped of cumulus before random allocation into: KSOM; KSOM-G; KSOM-G+0.1 μM DPI; KSOM+10 μM WY14643 in DMSO (1:10 000); KSOM+10 μM WY14643 in DMSO+0.1 μM DPI; KSOM+DMSO (1:10 000). After 20 min, embryos were washed free of DPI (flavoenzyme inhibitor) and returned to the same culture conditions without DPI. (At this time, embryos from each experimental group were randomly sampled and incubated for 30 min in 1 ml media containing 10 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate di-ester (CDCFDA) that fluoresces upon esterification to measure average total embryonic esterase activity.) After 1.5 or 3.5 h, DCHFDA (10 μM) was applied to each culture for 30 min, and then embryos were washed free of BSA and placed on glass coverslips in 10 μl BSA-free media. Green DCF or CDCF fluorescence from each embryo was quantified separately using a microfluorometer with filter excitation and emission wavelengths set at 460 and 510 nm respectively. Results were standardized by calculating the fluorescence ratio relative to the total average embryonic esterase activity.

Statistical analysis

Immunohistochemical expression levels were estimated from the pixel intensity of images using Image-J Software (National Institutes of Health, Bethesda, MD, USA). Embryos from three separate experiments underwent immuno-fluorescent staining at the same time, and all were analysed the next day using uniform confocal microscope settings to permit statistical analysis of staining intensity. Average grey level intensity from each embryo in each culture group was quantified with Image J, and mean grey levels per culture group per experiment were calculated. These data and those from measured ROS assays were compared by multi-variance ANOVA using Statgraphics Software. Values are presented as means $\pm$ S.E.M. for triplicate experiments.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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