Ca^{2+}-linked upregulation and mitochondrial production of nitric oxide in the mouse preimplantation embryo

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Summary

Previous studies have demonstrated a role for the signalling agent nitric oxide in regulating preimplantation embryo development. We have now investigated the biochemical mode of action of nitric oxide in mouse embryos in terms of mitochondrial function and Ca^{2+} signalling. DETA-NONOate, a nitric oxide donor, decreased day 4 blastocyst cell number and oxygen consumption, consistent with a role for nitric oxide in the inhibition mitochondrial cytochrome c oxidase. Using live cell imaging and the nitric-oxide-sensitive probe DAF-FM diacetate, nitric oxide was detected at all stages of preimplantation development and FRET analysis revealed a proportion of the nitric oxide to be colocalised with mitochondria. This suggests that mitochondria of preimplantation embryos produce nitric oxide to regulate their own oxygen consumption. Inhibiting or uncoupling the electron transport chain induced an increase in nitric oxide and [Ca^{2+}]$_i$ as well as disruption of Ca^{2+} deposits at the plasma membrane, suggesting that mitochondrial disruption can quickly compromise cellular function through Ca^{2+}-stimulated nitric oxide production. A link between antimycin-A-induced apoptosis and nitric oxide signalling is proposed.

Key words: Embryo, Nitric oxide, Mitochondria, Metabolism, Calcium

Introduction

Nitric oxide (NO) is a simple diatomic gas that functions as a cell signalling molecule but at high concentrations can be toxic (Moncada et al., 1991). NO is produced by the enzyme nitric oxide synthase (NOS) from arginine, an amino acid consumed by mouse, pig and human blastocysts (Lamb and Leese, 1994; Houghton et al., 2002; Sturmey and Leese, 2003). Three isoforms of NOS have been identified and characterised: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS); each displaying a cell-specific pattern of expression and different affinity for calmodulin (Kobzik et al., 1995; Mayer and Hemmens, 1997; Giulivi, 2003). A fourth isoform, mitochondrial NOS (mtNOS) has recently been reported (Ghafourifar and Richter, 1997) but its full characterisation is still in progress.

NO can modulate signal transduction via multiple pathways, depending on the local concentration and redox environment (Hanafy et al., 2001). These pathways include inhibition of mitochondrial respiration at the level of cytochrome c oxidase (Brown and Cooper, 1994; Cleeter et al., 1994) and initiation or inhibition of apoptosis, depending on concentration, source and cell type (Ceneviva et al., 1998; Pinsky et al., 1999; Li et al., 2000; Kim et al., 2001).

During mouse preimplantation embryo development, the fertilised one-cell embryo develops into a blastocyst, a process taking 4-5 days in vitro. NO is obligatory for these early stages of development (Gouge et al., 1998; Chen et al., 2001; Manser

et al., 2004) but high concentrations produced by NO donors are detrimental (Barroso et al., 1998; Lim and Hansel, 1998), suggesting that NO levels must be strictly regulated. The mRNA of all three NOS isoforms are present during preimplantation mouse development (Tranguch et al., 2003) whereas iNOS and eNOS proteins are detected at the blastocyst stage (Gouge et al., 1998; Nishikimi et al., 2001), supporting a role for NO production in blastocyst development.

Oxygen consumption, the best global indicator of the ability of an embryo to produce ATP, is low during the early preimplantation stages but increases four- to fivefold upon cavitation (Houghton et al., 1996). The mechanisms underlying this increase are unclear. Recently, however, inhibition of endogenous NO production with a NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) was found to cause an increase in blastocyst oxygen consumption (Manser et al., 2004). Moreover, blastocysts cultured in the presence of amino acids (including 0.2 mM arginine) consumed significantly less oxygen than those cultured with glutamine alone (Manser et al., 2004). Taken together, these findings suggest that NO can regulate blastocyst oxygen consumption, potentially via inhibition of cytochrome c oxidase, and that arginine availability may determine NO production and hence the degree of respiratory inhibition. However, the influence of exogenous NO on blastocyst oxygen consumption has not been investigated.

It has been proposed that mitochondria might produce their

own supply of NO to allow the rapid, local modulation of cell respiration (Ghafourifar and Cadenas, 2005). Indeed, endogenous mitochondrial NO production was first described in liver (Ghafourifar and Richter, 1997) and has subsequently been reported in other tissues (Elfering et al., 2002). The sequencing of a mitochondrial NOS isoform from rat liver (Elfering et al., 2002) and its immunogold localisation to the inner mitochondrial membrane (Lacza et al., 2001) establishes mtNOS as a regulator of mitochondrial function, although the existence of mtNOS has since been contested (Lacza et al., 2006).

Mitochondria of mouse preimplantation embryos are maternally derived and undergo a maturation process during development (Hillman and Tasca, 1969). By the blastocyst stage, a mixed population of immature and mature mitochondria are present in each blastomere (Stern et al., 1971) corresponding to a rise in metabolic activity and coinciding with an increase in the mitochondrial membrane potential ($\Delta \psi_m$) of a subset of mitochondria (Acton et al., 2004). The signalling processes underlying the metabolic activity of mitochondria in early embryos are unknown. As a regulator of oxygen consumption, mitochondrial NO production has the potential to act as a local modulator of mitochondrial function. However, there are no reports of mtNOS in the preimplantation embryo and the sites of NO production are unknown.

We have therefore examined the effect of a NO donor on the development and oxygen consumption of mouse blastocysts. Live-cell imaging using the NO-sensitive fluorescent probe 3-amino,4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) has been used to investigate the sites of NO production and fluorescence resonance energy transfer (FRET) was used to investigate the possibility of colocalisation of NO with mitochondria. In addition, the effect of chemical modulation of the electron transport chain and the involvement of Ca^{2+} in NO signalling in the two-cell mouse embryo have been explored.

Results

The effect of inhibiting NO production and increasing exogenous NO concentration on the rate of mouse blastocyst development was determined in the presence and absence of amino acids. An average of 49.8±3.4% of embryos cultured from the one-cell stage in potassium simplex optimisation medium (KSOM) supplemented with amino acids (KSOMaa) reached the blastocyst stage by Day 4 post fertilisation, which was not significantly different to 44.7±2.4%, 44.1±2.4% and 47.4±1.3% for L-NAME, 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (NONOate) and both chemicals together, respectively. However there was a significant reduction in cell number from 51.8±2.6 in KSOMaa to 41.6±3.4 ($P<0.05$), 40.5±1.4 ($P<0.001$) and 36.9±1.8 ($P<0.001$) with NONOate, L-NAME and both compounds together, respectively.

By day 4, 53±1.1% of zygotes cultured in KSOM (i.e. without supplemented amino acids) reached the blastocyst stage and the presence of 10 μM NONOate did not significantly alter this figure (50.9±2.0%). When cultured with 1 mM L-NAME, 0% of embryos reached the blastocyst stage by day 4 but the inclusion of 10 μM NONOate in addition to 1 mM L-NAME increased the blastocyst rate to 18±1.9%.

The oxygen consumption of Day 4 blastocysts cultured in KSOMaa was 0.24±0.02 nl/embryo/hour (Fig. 1). In the
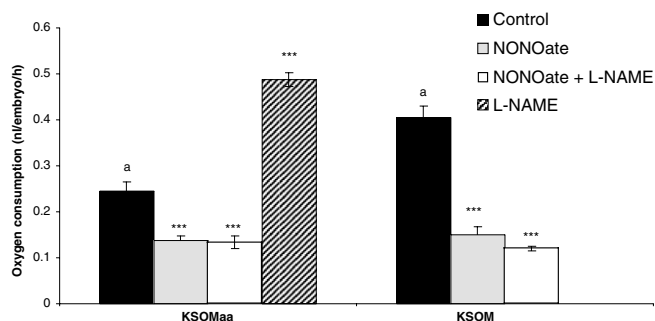
Fig. 1. Oxygen consumption of day 4 blastocysts cultured in KSOM or KSOMaa with or without 10 μM NONOate and/or 1 mM L-NAME. The number of determinations is shown in parentheses. Values are mean ± s.e.m. Significant differences in oxygen consumption in the various solutions were observed compared with levels in the relevant control (***$P<0.001$) and between the two control cultures in KSOM and KSOMaa ([a]$P<0.001$).

presence of 10 μM NONOate, oxygen consumption decreased significantly to 0.14±0.01 nl/embryo/hour whereas in the presence of 1 mM L-NAME, oxygen consumption was increased to 0.49±0.01 nl/embryo/hour ($P<0.001$). With the inclusion of both 1 mM L-NAME and 10 μM NONOate the oxygen consumption was reduced to 0.13±0.01 nl/embryo/hour, $P<0.001$ (Fig. 1). In KSOM, the oxygen consumption of day 4 blastocysts was significantly reduced from 0.40±0.03 nl/embryo/hour to 0.15±0.02 nl/embryo/hour in the presence of 10 μM NONOate ($P<0.001$) and to 0.12±0.01 nl/embryo/hour with both 10 μM NONOate and 1 mM L-NAME ($P<0.001$) (Fig. 1).

The NO-sensitive fluorescent probe, DAF-FM DA was used to detect NO production in preimplantation embryos. Although DAF-FM DA is fairly specific for NO, it has been suggested that the fluorescence intensity of this probe can be influenced by peroxynitrite (Roychowdhury et al., 2002), oxidants (Jourd'heuil, 2002), divalent cations, particularly Ca^{2+}, and light (Broillet et al., 2001). To ensure that the detected fluorescence was due to NO production and not to any interference, a number of control experiments were performed: DAF-FM fluorescence intensity was not altered in the presence of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) (FeTPPS, a peroxynitrite scavenger) or superoxide dismutase (SOD, a superoxide scavenger) (data not shown); the Ca^{2+} chelator, EGTA, caused a reduction in basal fluorescence but performing the experiment in Ca^{2+}-free medium did not alter the level of fluorescence (data not shown); and the fluorescence intensity of DAF-FM-stained embryos subjected to high-power laser during FRET experiments (see below) did not change, suggesting that DAF-FM is specific for NO.

Using laser-scanning confocal microscopy (LSCM) in combination with DAF-FM DA, endogenous NO production was detected throughout mouse preimplantation development but at a much reduced level in the large blastomere of zygotes (Fig. 2). DAF-FM fluorescence appeared to be uniform during the earlier stages, except for the polar body where a much stronger fluorescent signal was observed. In blastocysts, a higher level of NO production was detected in the epithelial trophectoderm (TE) relative to the inner cell mass (ICM) (Fig. 2f) and in some cases, single cells of exceptional
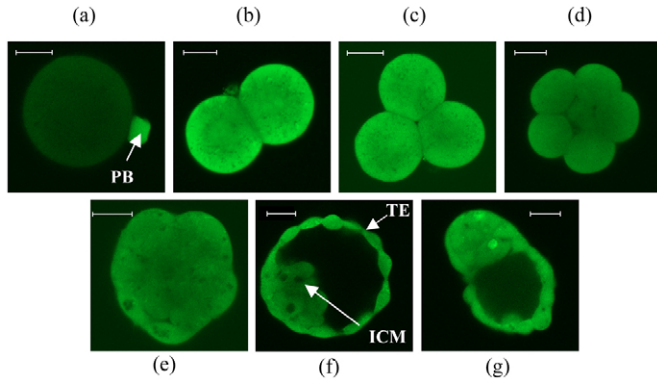
Fig. 2. In situ production of NO throughout preimplantation development detected using DAF-FM. (a) zygote, (b) two-cell, (c) four-cell, (d) eight-cell, (e) morula, (f) blastocyst, (g) in vivo-derived mouse blastocyst. PB, polar body; ICM, inner cell mass; TE, trophectoderm, Bars, 20 μm.

brightness were observed (Fig. 2g). Following membrane permeabilisation of two-cell embryos with the non-ionic detergent, digitonin, to remove nuclear and cytoplasmic DAF-FM, NO was found to aggregate in punctate spots, which when labelled with Mitotracker Deep Red (MT), colocalised with mitochondria (Fig. 3). It is possible that this apparent colocalisation was a function of the resolution limit of the confocal microscope, which is in the order of 500 nm. FRET was therefore used to determine whether a close association of NO and mitochondria was present in two-cell embryos.

An increase in donor fluorescence was detected in the discrete area of acceptor photobleaching (Fig. 4) but not in adjacent regions. The results were corrected to account for the acquisitional bleach of DAF-FM that occurs during image capture. Quantitatively, a strong FRET signal
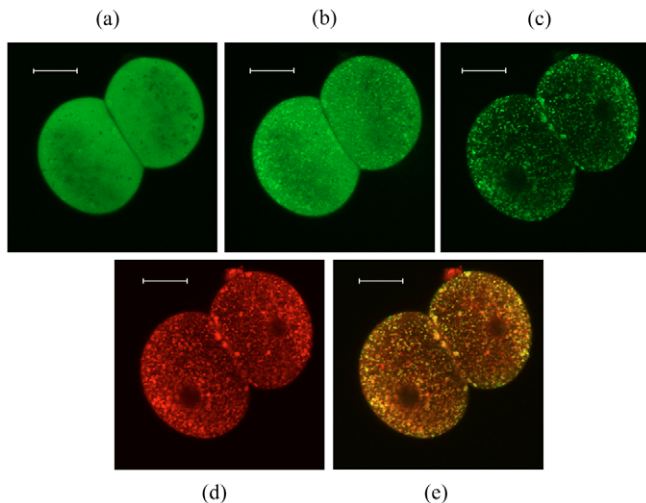
Fig. 3. A two-cell mouse embryo stained with DAF-FM and exposed to 10 μM digitonin to permeabilise cell and nuclear membranes. Images show DAF-FM staining (a) before, (b) after 1 minute, and (c) after 2 minutes of digitonin treatment. The punctate pattern revealed after release of cytoplasmic and nuclear DAF-FM into surrounding media appears to colocalise with mitochondria in the embryo stained with both DAF-FM and Mitotracker Deep Red (c,d,e). Bars, 20 μm.
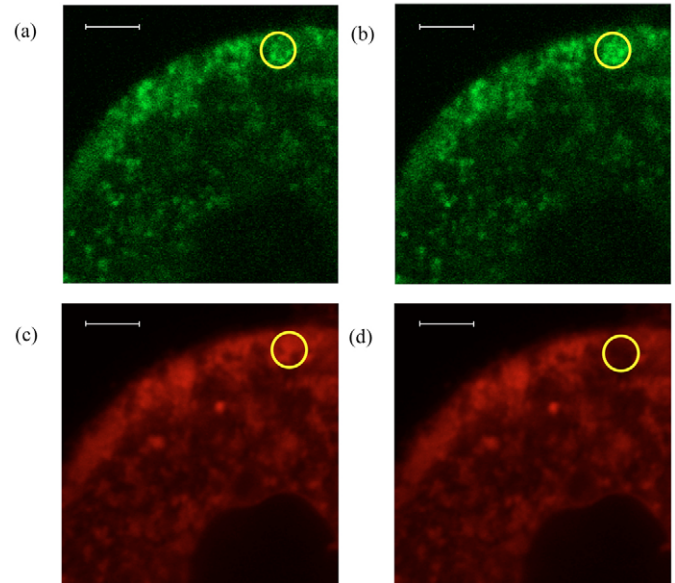
Fig. 4. Example of a FRET experiment. Two-cell mouse embryos were dual stained with DAF-FM and Mitotracker Deep Red. Images are those taken before (a,c) and after (b,d) photobleaching of the area indicated with the yellow circle. DAF-FM (a,b) and Mitotracker Deep Red (c,d) are separated for clarity. An increase in DAF-FM fluorescence is detected after photobleaching the selected area. Bars, 5 μm.

($Fd2/Fd1=1.20\pm0.02$, $P<0.0001$) was observed in the bleached area between DAF-FM and MT but when the donor or acceptor were omitted, photobleaching of a discrete area with the 633 nm laser line did not increase DAF-FM emission (Fig. 5). These results suggest that the increase in donor fluorescence intensity following photobleaching of the acceptor is due to the absence of the acceptor rather than a paradoxical reaction of the DAF-FM molecule or to background autofluorescence or bleed-through in response to exposure to 633 nm light. The data confirm the colocalisation of the two fluorescent probes and demonstrate a tight association between NO production and the mitochondria of mouse preimplantation embryos.

In the presence of 1 μM antimycin A or 100 μM carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP) the fluorescence intensity of DAF-FM in two-cell embryos was greatly increased relative to controls (Fig. 6, top panels). Two-cell embryos stained with 5 μM Fluo-3 AM to detect Ca^{2+} ions displayed a low level of fluorescence throughout each cell but high-intensity punctate staining was detected around the plasma membrane (Fig. 6, bottom left panels). When treated with 1 μM antimycin A or 100 μM FCCP, the fluorescence intensity was increased throughout both cells, especially in the polar body, and the high-intensity punctate staining in the plasma membrane could no longer be identified (Fig. 6, bottom right panels).

Discussion

The influence of an exogenous source of NO on mouse preimplantation development in KSOM and KSOMaa was assessed in terms of blastocyst formation, cell number and oxygen consumption. Confirming the work of Barroso et al. (Barroso et al., 1998), we found the rate of blastocyst formation

Fig. 5. Relative fluorescence of DAF-FM in the area selected for bleaching. Two-cell embryos stained with DAF-FM with or without Mitotracker Deep Red were subjected to photobleaching using the 633nm laser line and DAF-FM fluorescence quantified using laser-scanning confocal microscopy. Values are means ± s.e.m. and are corrected for acquisitional bleach and normalised for comparison. The number of determinations is shown in parentheses.
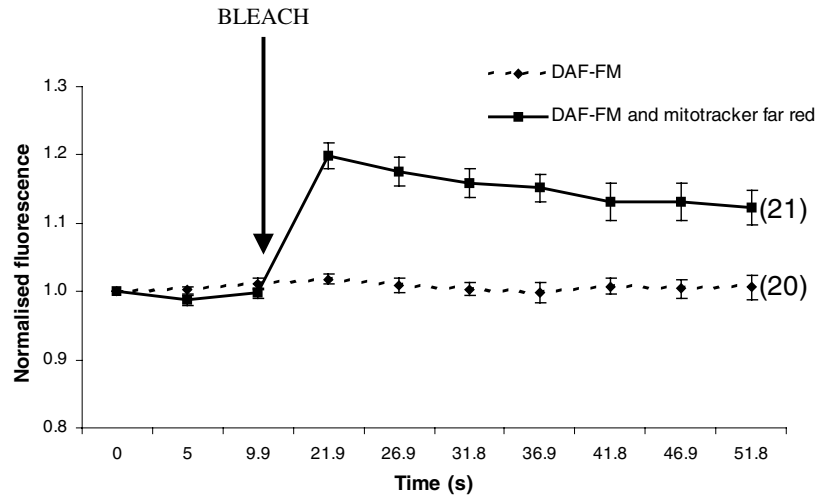
in the presence of a NO donor to be unchanged. However, the reduced cell number found in the presence of L-NAME and/or NONOate indicates that both a decrease and an increase in NO

Fig. 6. Two-cell mouse embryos stained with DAF-FM or Fluo-3 AM in the presence (+) or absence (–) of 1 μM antimycin A (AMA) or 100 μM FCCP. Bars, 20 μm.

compromises blastocyst quality. Although culturing preimplantation embryos with NO donors has been shown to stimulate apoptosis (Chen et al., 2001; Tranguch et al., 2003), an effect that might be mediated by enhanced $ONOO^-$ production, reducing NO production with a NOS inhibitor had no effect on apoptosis (Tranguch et al., 2003). As such, the decrease in cell number found in the presence of NONOate may be due to an increase in the incidence of apoptosis but with L-NAME, the reduction in cell number is unlikely to be explained by a change in the apoptotic rate.

Oxygen consumption provides the best global indication of the ability of an embryo to produce ATP and increases four to five times on formation of the mouse blastocyst when the majority of ATP is formed via oxidative phosphorylation (Houghton et al., 1996; Houghton and Leese, 2004). The proportion of oxygen used by mitochondria also changes during early embryo development from 30% before cavitation to 70% once the blastocyst is formed (Trimarchi et al., 2000). Despite the major role played by oxidative phosphorylation throughout preimplantation development, mechanisms underlying the regulation of oxygen consumption by embryos either via oxidative phosphorylation or by other processes, are not known.

We have previously demonstrated that decreasing endogenous NO production with L-NAME causes a significant increase in blastocyst oxygen consumption, most probably owing to relief of basal inhibition of cytochrome c oxidase by NO (Manser et al., 2004). The availability of amino acids including the NO precursor, arginine, also decreased oxygen consumption, probably owing to enhanced NO production and thus cytochrome c oxidase inhibition. We now demonstrate that exogenous NO is also capable of inhibiting blastocyst oxygen consumption and that provision of a NO donor can overcome the increase in oxygen uptake caused by L-NAME. Although it cannot be ruled out that the reduced cell number of blastocysts cultured with NONOate with or without L-NAME may be partly responsible for the lower oxygen consumption, embryos cultured with L-NAME alone also contained fewer cells but consumed
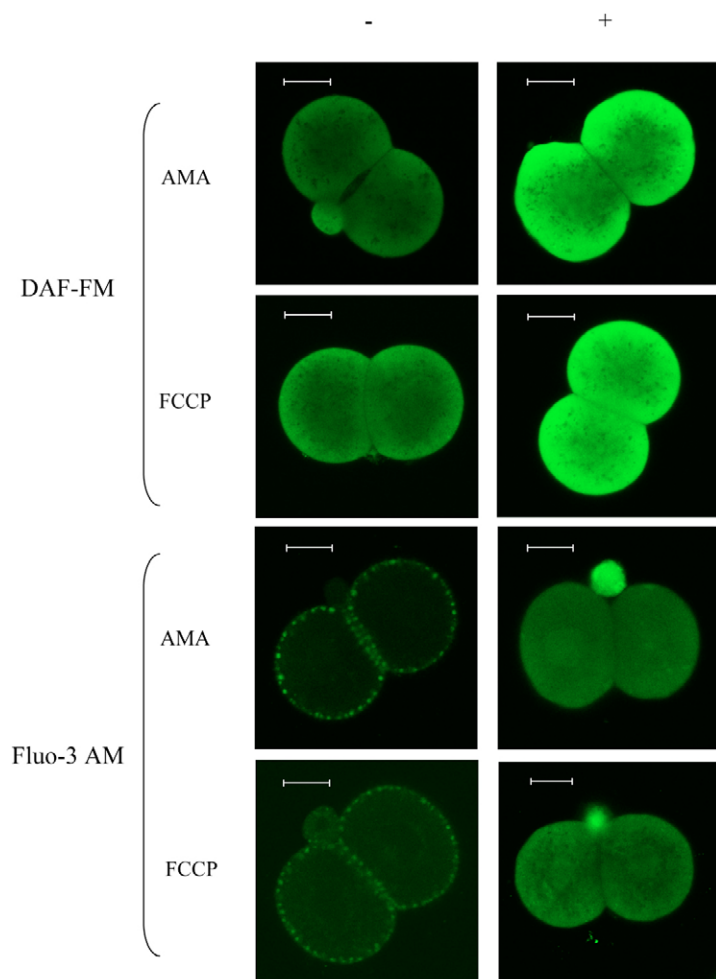
more oxygen. This suggests that a reduction in blastocyst cell number does not significantly contribute to the overall rate of oxygen consumption. We suggest that mitochondria are a target for NO and that the low rate of oxygen use demonstrated in both media in the presence of NONOate represents the oxygen used for non-mitochondrial processes. Whether this is by reversible inhibition of the electron transport chain by NO at the level of cytochrome c oxidase or the persistent inhibition of complex I, via $ONOO^-$-mediated oxidation of sulfhydryl groups (Riobo et al., 2001) has not been investigated. However, it is unlikely that NONOate inhibits the electron transport chain completely because ATP production via the Krebs cycle is essential for embryo development (Thomson, 1967; Auerbach and Brinster, 1968). It is plausible that NO produced either by the embryo or by surrounding cells of the reproductive tract is important for the regulation of blastocyst oxygen consumption and that elevated levels of NO in vivo may cause excessive inhibition of oxidative phosphorylation which could compromise development.

The NO-sensitive probe, DAF-FM DA was used to detect NO in mouse preimplantation embryos. Despite widespread use of this probe as an indicator of NO production, the specificity of DAF-FM, and the related probe DAF-2 for NO has been questioned. It has been suggested that DAF probes may react with peroxynitrite (Roychowdhury et al., 2002) and divalent cations, especially Ca^{2+} (Broillet et al., 2001) and that DAF fluorescence is enhanced upon illumination (Broillet et al., 2001) and in the presence of oxidants (Jourd'heuil, 2002). The properties of DAF-FM DA were recently tested and it was concluded that DAF-FM DA is fairly specific for NO under physiological conditions (Balcerczyk et al., 2005). To test this conclusion and avoid false positives we examined the specificity of DAF-FM in the two-cell embryo. DAF-FM fluorescence was detected throughout both cells of the two-cell embryo and the level was not altered by either FeTPPS or SOD, indicating that DAF-FM does not react with $ONOO^-$ or superoxide ($O_2^{.-}$). Since $O_2^{.-}$ can react with NO to form $ONOO^-$, reducing the $O_2^{.-}$ concentration with SOD can reduce $ONOO^-$ production and improve the bioavailability of NO under certain conditions (Liochev and Fridovich, 2002). However, one important consideration highlighted by the model of Liochev and Fridovich (Liochev and Fridovich, 2002) is the proportion of NO that is directed towards $ONOO^-$ formation in a particular system; if the majority of NO reacts with targets other than $O_2^{.-}$, and the production of NO exceeds that of $O_2^{.-}$, addition of SOD is likely to decrease $ONOO^-$ formation with no effect on NO production (Liochev and Fridovich, 2002). Since culturing embryos with SOD did not alter the fluorescence of DAF-FM, it is likely that the latter situation may prevail in the two-cell embryo; however, current techniques are not sufficiently sensitive to quantify NO production by embryos and this theory cannot as yet be verified.

The presence of EGTA caused a reduction in DAF-FM fluorescence, but performing the experiment in Ca^{2+}-free medium had no effect, suggesting that DAF-FM does not react with Ca^{2+} per se, but rather that Ca^{2+} chelation inhibits basal NO production via Ca^{2+}-dependent eNOS. The potential problem of light sensitivity was addressed during the FRET experiments where the negative-control embryo, stained with DAF-FM, was subjected to high-power laser (488 nm) with no

increase in fluorescence. Having confirmed the specificity of DAF-FM DA, NO production was detected throughout mouse preimplantation embryos of all stages, although a much-reduced level was observed in zygotes. In good quality embryos before cavitation, the production of NO was uniform in all blastomeres. Higher levels of fluorescence were detected in embryos of inferior quality, particularly in cells likely to be undergoing degradation, and in the polar body. Since DNA damage and apoptosis have been shown to result from culturing embryos with NO donors (Chen et al., 2001; Tranguch et al., 2003), it is tempting to speculate that the enhanced NO production detected in particular cells and in the polar body is involved in the process of cell death in targeted blastomeres of the preimplantation embryo. This could be of particular interest in the context of the polar body because this cell undergoes degradation but the underlying mechanisms are unknown.

At the blastocyst stage, NO production was detected in all cells but a higher level was found in the TE relative to the ICM. To ensure that laser penetration was not responsible for reduced ICM fluorescence, we examined mid-plane sections of morulae. We found no variation in signal from the innermost cells of morulae compared with those at the edge, suggesting that laser penetration was not a contributory factor. By contrast, Nishikimi et al. detected increased NOS activity in the ICM compared with the TE of early blastocysts using NADPH-diaphorase staining, and in the expanded blastocyst, found NOS activity to be confined entirely to the ICM (Nishikimi et al., 2001). Several factors could account for this anomaly. Using biochemical characterisation, neuronal NADPH-diaphorase has been equated with NOS (Hope et al., 1991) and NADPH-diaphorase histochemistry has historically been used to identify NOS activity. However, there is evidence (1) that not all NADPH-diaphorase activity is due to NOS (Worl et al., 1994) and (2) that some NOS does not exhibit NADPH-diaphorase activity (Wang et al., 1997; Doyle and Slater, 1997), raising questions as to the specificity of this technique for detecting all generic NOS activity. In addition, Nishikimi et al. cultured embryos in KSOM (Nishikimi et al., 2001) whereas in the present study with DAF-FM, KSOM was supplemented with amino acids. We have already demonstrated that amino acid supplementation can influence blastocyst oxygen consumption, potentially by enhanced NO production from arginine (Manser et al., 2004). The majority of mitochondria in blastocysts are in the TE, which is metabolically more active than the ICM (Houghton, F. D., 2006). eNOS and iNOS proteins are both found in the TE, and iNOS expression is greater in the TE of KSOM-cultured blastocysts compared with the ICM (Nishikimi et al., 2001). This finding implies that NO production may be stimulated in the TE and we suggest that culture in amino acids results in enhanced NO production in the TE to inhibit mitochondrial oxidative phosphorylation, reduce oxygen consumption and in this way protect against oxidative damage.

The proposition that mitochondria may be capable of producing their own supply of NO has recently generated significant interest. Although some still contest the existence of a mitochondrial isoform of NOS (Lacza et al., 2006), increasing evidence points to the rapid, local regulation of oxidative metabolism, membrane potential and apoptosis being achieved via NO production in mitochondria (Ghafourifar and Cadenas, 2005). Moreover, the sequence of mtNOS has been

obtained from rat liver mitochondria (Elfering et al., 2002) and the Ca²⁺-stimulated production of NO from neuronal mitochondria has been demonstrated convincingly (Dedkova et al., 2004).

The mitochondria of preimplantation embryos undergo maturation from the point of fertilisation onwards, characterised by an increase in the number of cristae and a slow change towards an elongated shape (Stern et al., 1971). Mitochondrial activity, in terms of elevated $\Delta\psi_m$, increases as cell division occurs, to ensure that the energy requirement of each blastomere is fulfilled despite decreasing numbers of mitochondria per cell (Acton et al., 2004).

There is colocalisation of NO and mitochondria in two-cell embryos and in the TE of blastocysts. The colocalisation was particularly obvious at the cleavage stage owing to the larger cell size and reduced level of chromatic aberration. This mitochondrial NO is of particular interest in light of the expression pattern of iNOS in early embryos (Nishikimi et al., 2001). It is generally accepted that mtNOS is a NOS isoform distinct from iNOS, eNOS and nNOS (Lacza et al., 2003), but iNOS has been shown to be essential for the regulation of mitochondrial function in mouse hepatic cells (Venkatraman et al., 2004). The punctate expression pattern of iNOS detected in preimplantation embryos (Nishikimi et al., 2001) may indicate that iNOS is localised close to mitochondria in order to influence their activity. Alternatively, the antibody used for detection of iNOS may have cross-reacted with mtNOS as a result of sequence homology, as has previously been shown to occur in purified rat liver mitochondria (Tatoyan and Giulivi, 1998). Such immunocytochemistry experiments would need to be repeated with different antibodies in combination with a mitochondrial stain to investigate these possibilities further.

The observation that NO is found within mitochondria of preimplantation embryos raises important questions regarding the potential purpose of such a mechanism; NO may attenuate oxygen tension gradients within tissues and allow cells furthest away from blood capillaries access to an adequate supply of oxygen. Since it has been shown using a mathematical model that the centre of the preimplantation embryo could become marginally hypoxic (Byatt-Smith et al., 1991), NO may decrease the oxygen consumption of blastomeres close to the zona pellucida to allow sufficient oxygen to diffuse to cells at the centre. Alternatively, NO may be acting in a protective manner to prevent excess production of free radicals in vitro, since the oxygen tension (20%) is 3-4 times greater than that of the oviduct (Fischer and Bavister, 1993) and the production of free radicals is enhanced (Goto et al., 1993).

Aside from mitochondrial NO production, inhibiting complex III of the mitochondrial electron transport chain with antimycin A resulted in a large, general increase in NO production in both blastomeres of the two-cell embryo. A similar effect has been found in RAW 264.7 macrophages where inhibition of either complex I or complex III of the electron transport chain with rotenone or antimycin A, respectively, resulted in enhanced production of NO, and uncoupling the electron transport chain using FCCP resulted in a reduction in NO production (Tirosh et al., 2001). The authors proposed that mitochondria can regulate NAD(P)H concentrations in the vicinity of cytosolic iNOS and that electron transport chain inhibitors and uncouplers cause accumulation and depletion, respectively, of cofactors regulating iNOS activity. However, in the two-cell embryo we found that both antimycin A and FCCP significantly increased NO production suggesting that the mechanism in this case was not related to excess or depleted co-factors. Instead we hypothesise that the increase in NO with antimycin A and FCCP was due to a common factor between the two treatments, such as a depleted mitochondrial membrane potential, which stimulates an increase in intracellular Ca²⁺ concentration and thus NO production by means of a Ca²⁺-dependent NOS isoform (eNOS or mtNOS). To test this proposition, embryos were stained with Fluo-3, a Ca²⁺-sensitive probe, in the presence and absence of antimycin A or FCCP. Identification of the punctate pattern of Ca²⁺ staining around the plasma membrane of each cell in control embryos was investigated using caveolin-1 immunofluorescence to detect caveolae and fluorescein isothiocyanate-lens culinaris agglutinin to localise cortical granules. Neither staining technique highlighted areas similar to those detected with Fluo-3 (data not shown). However, antimycin A and FCCP completely disrupted the plasma membrane Ca²⁺-rich vesicles and caused a general increase in Ca²⁺ concentration throughout both cells. Treatment of HL-60 cells with antimycin A has previously been shown to stimulate apoptosis (King and Radicchi-Mastroianni, 2002) but the mechanism underlying this process does not involve stimulation of the intrinsic apoptotic pathway usually associated with mitochondrial dysfunction (King, 2005). As discussed above, NO can stimulate apoptosis in many cell types (Brune, 2005) and sodium nitroprusside (SNP) induces apoptosis in mouse blastocysts (Chen et al., 2001). It is tempting to hypothesise that one of the mechanisms underlying antimycin A-induced apoptosis may be increased NO production owing to disruption of plasma membrane Ca²⁺ signalling and a general increase in the concentration of free Ca²⁺.

In conclusion, we have demonstrated that the oxygen consumption of mouse blastocysts is decreased in the presence of a NO donor, suggesting that NO can modulate the respiratory rate of the preimplantation embryo, potentially by means of cytochrome c oxidase. We have also shown that mitochondria of cleavage-stage embryos have their own supply of NO; a mechanism that may be important in regulating mitochondrial activity to suit the changing needs of the embryo as it moves through the dynamic environment of the reproductive tract. In addition, we have found that chemically modulating the electron transport chain results in a large increase in NO production, disruption of plasma membrane Ca²⁺ signalling and an increase in Ca²⁺ concentration in two-cell embryos. The possibility that the mechanism of antimycin-A-induced apoptosis may involve increased NO production by means of an enhanced intracellular Ca²⁺ concentration warrants further investigation.

Materials and Methods

Materials
All chemicals were obtained from Sigma, UK unless otherwise stated.

Embryo production and recovery
Virgin mice, 6- to 8-weeks old, of the strain CBA/Ca × C57BL/6 were superovulated by intraperitoneal injection of 5 IU (0.1 ml) pregnant mare's serum gonadotrophin (Folligon, Intervet, UK), followed 48 hours later by 5 IU (0.1 ml) human chorionic gonadotrophin (Chorulon, Intervet, UK). Females were placed with F1 males of the same strain, and the presence of a vaginal plug the following morning (day 1) was taken as an indication that mating had occurred.

Mice were killed by cervical dislocation. Zygotes were retrieved from the oviducts and cultured under oil in groups of 10-15 to the blastocyst stage in 10 µl drops of KSOM (Lawitts and Biggers, 1993) or KSOMaa (Tay et al., 1997). Media were supplemented with 1 mM L-NAME or 10 µM NONOate, as appropriate. NONOate is a NO donor that spontaneously releases NO in a rate-controlled manner ($T_{1/2}$ in solution 56.7 hours), making it ideal for studies where a continuous supply of NO over a relatively long period is required. The number of embryos reaching the blastocyst stage on day 4 was recorded.

Cell counts
The zona pellucida was removed using acid Tyrode's solution, pH 2.1, and embryos were incubated in 50 µg/ml Hoechst 33428 at 2-4°C for at least 1 hour. Embryos were washed in absolute alcohol for 5-30 minutes before being mounted in a drop of glycerol on a microscope slide beneath a coverslip. The number of cells in each embryo was counted using a fluorescence microscope (Vickers, York, UK), with filter block A.

Oxygen consumption measurement
The oxygen consumption of preimplantation embryos was performed as previously described (Houghton et al., 1996). Briefly, 1 µl of 1 mM pyrene, dissolved in paraffin oil (BDH, UK), was drawn into a 5 µl polymerase chain reaction (PCR) micropipette (Fisher Scientific, UK). Groups of 10-15 blastocysts in 2 µl HEPES buffered KSOM(aa) [KSOM(aa)H] with or without 1 mM L-NAME and/or 10 µM NONOate as appropriate, were sealed in tubes. Oxygen consumption was measured by following the increase in pyrene fluorescence at 340 nM over a 3-4 hour period, using a Fluovert fluorescence microscope (Leica, Milton Keynes, UK) with photomultiplier and photometer attachments at 37°C. Two control tubes were measured concurrently; a 0% oxygen control containing 1 µl pyrene and 2 µl yeast (1 mg/ml) in 60 mM glucose, pre-equilibrated overnight, and a 20% oxygen control containing 1 µl pyrene and 2 µl KSOMaaH in the absence of embryos. Arbitrary fluorescence readings were converted to values for oxygen consumption in nl/embryo/hour using a computerised mathematical model (Houghton et al., 1996).

Imaging of preimplantation embryos
All images were acquired on a Zeiss LSM 510 Meta confocal system with a Zeiss Axiovert inverted microscope. Embryos were secured to poly-L-lysine-coated imaging chambers in KSOMaaH and viewed with a 63× (1.4 NA) oil DIC lens at room temperature.

DAF-FM DA
Embryos were incubated with 5 mM EGTA, 100 µM FCCP, 100 µM FeTPPS, 700 U/ml SOD or 1 µM antimycin A (15 minutes). Embryos were loaded with 10 µM DAF-FM DA (Molecular Probes, UK) with or without the necessary chemicals for 40 minutes at 37°C before a 15 minute wash in KSOMaaH with or without chemical additions. DAF-FM was excited at 488 nm using an HFT dichroic mirror and imaged through a 505-530 nm band-pass filter.

Permeabilised embryos
The zona pellucida was removed using acid Tyrode's solution, pH 2.1, and embryos incubated with 10 µM DAF-FM DA (1 hour, 37°C) followed by 1 µM Mitotracker Deep Red 633 (MT, Molecular Probes, UK) for 15 minutes. Embryos were washed in KSOMaaH (15 minutes, 37°C) before imaging. The 488 nm laser and HFT dichroic mirror was used to excite both probes and fluorescent signals were collected using a 505-530 band-pass filter (DAF-FM) and 650 long-pass filter (MT). This simultaneous imaging protocol was used to reduce the chromatic aberration detected when using sequential imaging (described below for FRET). Images were taken before, during and after treatment with 10 µM digitonin (2 minutes). Embryos were washed with KSOMaaH to remove digitonin following membrane permeabilisation.

FRET
FRET is a distance-dependent interaction; for energy transfer to occur, donor and acceptor molecules must be in close proximity (typically 1-10 nm). FRET was measured using a method developed for LSCM (McLean et al., 2000; Rocheville et al., 2000). Our experimental design assessed FRET by measuring the 'donor' fluorescence, DAF-FM, before (Fd1) and after (Fd2) complete photobleaching of the 'acceptor', MT. The ratio of Fd2:Fd1 equals 1.0 in the absence of FRET. If Fd2:Fd1>1.0, the fluorophores must be within 10 nm. Although the published excitation and emission spectra of DAF-FM and MT are quite far from each other which might suggest that these two probes would be unlikely to undergo FRET, in our system the MT probe could be easily excited by the 488nm laser (simultaneous imaging protocol above) and as such, the spectra overlap sufficiently to allow energy transfer between the two probes.

Permeabilised embryos stained with DAF-FM, MT or both, were imaged using the 488 nm laser to excite DAF-FM (signal collected using a 505-530 band-pass filter). For each FRET image, a line average of two was used to prevent unnecessary acquisitional bleaching of samples. A second scan was performed with the 633 nm laser to excite MT (signal collected using a 650 long-pass filter) in accordance with a sequential-imaging protocol. Three images were taken with each laser to

determine acquisitional bleach and a small section (~3×3 µm) was photobleached with intense 633 nm light to destroy the acceptor molecule, MT. The embryo was re-scanned several times with both lasers and the degree of FRET monitored.

Ca^{2+} imaging
Embryos were pre-incubated with KSOMaaH with or without 1 µM antimycin A (15 minutes) before 5 µM Fluo-3 AM (Molecular Probes, UK) with or without 1 µM antimycin A (40 minutes, 37°C). Embryos were washed in KSOMaaH with or without antimycin A and imaged. Fluo-3 was excited at 488 nm using an HFT dichroic mirror and imaged through a 505-530 nm band-pass filter.

Statistical analysis
Oxygen consumption data, expressed in terms of nl/embryo/hour and cell counts were analysed by one-way analysis of variance. Differences between individual means were compared by Fisher's test. When comparing developmental rates, the average percentage and s.e.m. of embryos at each developmental stage by day 4 was calculated. One-way analysis of variance and Fisher's tests were performed following angular transformation.

For FRET calculations, acquisitional bleach of DAF-FM in each area of interest was calculated based on the first three experimental scans (i.e. before bleaching). Subsequent fluorescence values were corrected to account for acquisitional bleach and each experiment was normalised to 1.0. The ratio of DAF-FM fluorescence following and before photobleaching in dual-stained samples was compared with the null hypothesis value of 1.0 (indicating no change) by a one-sample t-test. This ratio was also compared with the ratio of DAF-FM fluorescence following and before photobleaching for samples stained only with DAF-FM using a two-sample t-test.

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