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Fetal programming alters reactive oxygen species production in sheep cardiac mitochondria

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Abstract

Exposure to an adverse intrauterine environment is recognized as an important risk factor for the development of cardiovascular disease later in life. Although oxidative stress has been proposed as a mechanism for the fetal programming phenotype, the role of mitochondrial O₂^{•−} (superoxide radical) production has not been explored. To determine whether mitochondrial ROS (reactive oxygen species) production is altered by *in utero* programming, pregnant ewes were given a 48-h dexamethasone (dexamethasone-exposed, 0.28 mg·kg^{−1} of body weight·day^{−1}) or saline (control) infusion at 27–28 days gestation (term = 145 days). Intact left ventricular mitochondria and freeze-thaw mitochondrial membranes were studied from offspring at 4-months of age. AmplexRed was used to measure H₂O₂ production. Activities of the antioxidant enzymes Mn-SOD (manganese superoxide dismutase), GPx (glutathione peroxidase) and catalase were measured. Compared with controls, a significant increase in Complex I H₂O₂ production was found in intact mitochondria from dexamethasone-exposed animals. The treatment differences in Complex I-driven H₂O₂ production were not seen in mitochondrial membranes. Consistent changes in H₂O₂ production from Complex III in programmed animals were not found. Despite the increase in H₂O₂ production in intact mitochondria from programmed animals, dexamethasone exposure significantly increased mitochondrial catalase activity, whereas Mn-SOD and GPx activities were unchanged. The results of the present study point to an increase in the rate of release of H₂O₂ from programmed mitochondria despite an increase in catalase activity. Greater mitochondrial H₂O₂ release into the cell may play a role in the development of adult disease following exposure to an adverse intrauterine environment.

Keywords

antioxidant; catalase; hydrogen peroxide; ovine myocardium; superoxide

INTRODUCTION

The concept that an adverse intrauterine environment can affect the risk of developing cardiovascular disease later in life was initially suggested by epidemiological studies. In

their seminal work, Barker et al. [1] identified that children born at a lower birthweight had more than a 2-fold increase in the prevalence of coronary artery disease as adults [1]. Subsequent studies in different populations [2,3] confirmed the initial epidemiological findings of Barker et al. [1]. More recently, the development of several animal models has demonstrated that an insult during fetal development can have lasting effects on both a physiological phenotype and on gene expression. Developmental programming of adult cardiovascular disease models that have proven useful are the maternal low-protein diet in rats [4] and a 48-h steroid infusion in the first trimester in sheep [5]. In each of these models, the programmed animals are born without an abnormal cardiovascular phenotype but develop hypertension and cardiac hypertrophy by 4–6 months of age.

There is emerging evidence that altered ROS (reactive oxygen species) production may play a role in the cardiovascular phenotype found in developmental programming. Studies in the sheep model have identified that the production of ROS is increased in the coronary arteries of programmed animals [6]. There is direct evidence that ROS play a role in altered cardiac function of developmentally programmed rats. Studies by Elmes et al. [7] demonstrated that adult rat hearts born following a low-protein diet during pregnancy had an increased susceptibility to ischaemia/reperfusion. This same group studied a similar set of animals following manipulation of myocardial antioxidant status [8]. Enhancing ROS scavenging pathways in the programmed hearts resulted in improved recovery following ischaemia and reperfusion.

Through one-electron reductions of O_2 from the mitochondrial electron-transport chain, mitochondria are becoming increasingly recognized as an important source of ROS, including $O_2^{\bullet-}$ (superoxide radical) and H_2O_2 [9,10]. Complex I and Complex III have been identified as the two primary locations in the electron-transport chain where one-electron reductions of O_2 occur [11,12]. Reduction of the NADH dehydrogenase site of Complex I results in $O_2^{\bullet-}$ production that is directed toward the mitochondrial matrix [11,13]. Complex III can generate ROS at both the Q_o and Q_i sites and release ROS into the inner membrane space or mitochondrial matrix respectively [11,12]. There is growing evidence that mitochondrial ROS production can cause direct mitochondrial damage or cellular dysfunction through oxidation of key structural and metabolic proteins [14]; however, the role that ROS derived from the electron-transport chain play in the developmental programming phenotype has not been explored. The goals of the present study were to examine the mitochondrial sources of ROS in programmed sheep cardiac mitochondria and to test the hypothesis that fetal exposure to steroids causes increased postnatal mitochondrial ROS production.

MATERIALS AND METHODS

Animal model

The sheep model of developmental programming that was used for the present study has been used extensively in our laboratory [15,16]. Briefly, time-dated pregnant ewes were obtained locally and given either 0.28 mg kg^{-1} of body weight day^{-1} dexamethasone (dexamethasone-exposed) or saline (control) intravenously for 48 h at 27–28 days gestation (term = 145 days). Offspring were allowed to deliver and were maintained at a remote facility. Males were neutered at 1 week of age. On the day of study (at 21–25 weeks of age), the animals were anaesthetized with 12 mg of sodium thiopental (Abbott Laboratories)/kg of body weight, intubated and ventilated with a mixture of halothane (1%), oxygen (33%) and nitrous oxide (66%). Hearts were removed through a midline sternotomy and a portion of the left ventricular free wall was excised for mitochondrial isolation. All animals were handled in accordance with the regulations in the Animal Welfare Act and the National

Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols were approved by the University of Iowa Animal Care and Use Committee.

Mitochondrial isolation

Cardiac subsarcolemmal and interfibrillar mitochondria were isolated at 0–4°C using a modification of the technique of Saks et al. [17] as described previously [18]. Briefly, approx. 1 g of myocardium was minced with scissors and placed in MSE buffer [220 mM mannitol, 70 mM sucrose, 5 mM Mops and 2 mM EGTA (pH 7.2)]. Trypsin (2.8 mg) was added to the tissue suspension and incubated at 0°C for 15 min. The reaction was stopped with 3.8 mg of trypsin inhibitor dissolved in 20 ml of MSE buffer with 1 mg/ml BSA. After decanting the fluid, the tissue was resuspended in 20 ml of MSE buffer with BSA and homogenized with a Teflon-glass homogenizer. The homogenate was centrifuged three times at 600 *g* for 10 min, each time saving the supernatant. The mitochondria in the supernatant were then pelleted at 8000 *g* for 15 min and washed once in MSE buffer with BSA. The final pellet was resuspended in MSE buffer without BSA.

Mitochondrial oxygen consumption during State-3 activity (substrate and ADP excess) and the rate of oxygen consumption during State-4 activity (ATP present and ADP depleted) were measured in a temperature-regulated chamber with a Clark-type oxygen electrode. Mitochondria were continuously stirred during these measurements. Substrates used included either glutamate (5 mM) and malate (5 mM) or succinate (5 mM). In addition to the substrates, the mitochondrial suspension included 130 mM potassium chloride, 20 mM Hepes, 2.5 mM magnesium chloride, 0.5 mM EDTA and 5 mM potassium phosphate (pH 7.2). After stabilization of the suspension, State-3 activity was measured following addition of 580 μ M ADP. State-4 activity was then measured after depletion of available ADP.

Mitochondrial membrane preparation

Mitochondrial membranes that removed mitochondrial matrix proteins and allowed free exchange between the mitochondrial matrix and extramitochondrial space [19] were prepared by subjecting intact mitochondria to three freeze-thaw cycles in MSE buffer followed by centrifugation at 10 000 *g* for 10 min at 4°C. The pellet was washed once. The pellet of mitochondrial membranes was resuspended in MSE buffer and used in the studies described below. Electron microscopy of the mitochondrial membranes demonstrated complete disruption of the mitochondrial architecture, and immunoblot studies found a loss of Mn-SOD [manganese SOD (superoxide dismutase)] protein (results not shown).

Measurement of H₂O₂ production

The rate of H₂O₂ production by intact mitochondria and mitochondrial membranes was determined using AmplexRed reagent (20 μ M; Invitrogen) in the presence of 1 unit of HRP (horseradish peroxidase; Sigma–Aldrich) to generate the fluorescent compound resorufin [20]. Resorufin levels were measured using a NOVOSTar Microplate Fluorometer (BMG Labtech) with an excitation wavelength of 544 nm and emission wavelength of 590 nm. Reactions were performed using 50 μ g of intact mitochondria or mitochondrial membranes in buffer containing 130 mM potassium chloride, 20 mM Hepes, 2.5 mM magnesium chloride, 5 mM potassium phosphate and 0.5 mM EDTA, pH 7.2 with either the Complex I substrates 5 mM glutamate/5 mM malate or the Complex II substrate 5 mM succinate. When mitochondrial membranes were studied, 80 μ M NADH was added to provide reducing equivalents directly to Complex I. Other additions included 2 μ M rotenone to inhibit Complex I, 1.8 μ M antimycin A to inhibit Complex III, and/or 0.5 μ M FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) to collapse the mitochondrial membrane potential, oxidize the respiratory chain complexes and maximally stimulate flux through the electron-transport chain [21,22]. Reactions were followed for 3 min and the slope of the

final 2 min was calculated to determine the rate of H₂O₂ production. A standard curve was generated using H₂O₂ that was linear to 5 nmols of added H₂O₂. Addition of SOD to the reactions did not increase the measured amount of H₂O₂ production, whereas addition of catalase eliminated the signal.

Enzyme activities

Mitochondrial membranes were used to determine the activities of NADH/cytochrome *c* oxidoreductase as described by Hatefi and Rieske [23]. Briefly, to a solution of 20 mM potassium phosphate, 1 mM EDTA, 2 mM sodium azide and 0.06% cytochrome *c* (pH 8.0) at 38°C, 5 µg of mitochondrial membrane protein was added. The rate of reduction of cytochrome *c* at 550 nm was monitored for 4 min. Samples were run in quadruplicate and average values were used to determine enzyme activity.

Catalase and GPx (glutathione peroxidase) activities were determined as previously described [24,25] and expressed as milli-units/mg of protein as determined by the method of Lowry [25a]. Mn-SOD activity was determined spectrophotometrically as previously described [26] and expressed as units/mg of protein.

Quantitative immunoblots

Immunoblots were performed as described previously [27] using left ventricular myocardium from a group of animals other than those used for the isolated mitochondria studies. These animals ranged in age from 17 weeks to 23 weeks and included six control females, six control males, nine dexamethasone-exposed females and four dexamethasone-exposed males. Briefly, myocardium was homogenized in the presence of protease inhibitors, soya bean trypsin inhibitor, leupeptin and PMSF, in 50 mM Tris/HCl, 10mM EDTA, 150 mM NaCl and 0.1 % 2-mercaptoethanol, pH 7.5. Following centrifugation (12 000 *g* for 20 min at 4°C), the total protein content of the supernatant was quantified spectrophotometrically, and 20 µg of protein was separated by SDS/PAGE and then transferred on to a nitrocellulose membrane. Membranes were blocked with 5 % Li-Cor blocking reagent (Li-Cor Biotechnology) for 1 h and then incubated in primary antibody overnight at 5°C. Bound primary antibody was detected by incubation with IR-labelled secondary antibodies (IRDye 800 or IRDye 700 700DX; Li-Cor Biotechnology), read and quantified with a Li-Cor Odyssey Imaging System (Li-Cor Biotechnology).

Primary antibodies used included antibodies specific for Mn-SOD (sc-30 080 used at a dilution of 1:1000; Santa Cruz Biotechnology); the 39 kDa subunit of Complex I (20C11 used at a dilution of 1:200 000; Invitrogen) and the core 2 subunit of Complex III (13G12 used at a dilution of 1:200 000; Invitrogen).

Statistics

All results are expressed as means ± S.E.M. Statistical analyses were performed using SAS version 9.1. Comparisons between groups were performed using an unpaired Student's *t* test, two-way ANOVA and generalized linear modelling when appropriate. If the *F*-statistic of the ANOVA identified significant differences, post-hoc comparisons between means were made using the Bonferroni post-hoc test. Significant differences were identified at the *P* < 0.05 level.

RESULTS

Mitochondrial oxidative phosphorylation

For the mitochondrial studies in the present study, control animals (*n* = 9) were 22.6 ± 0.6 weeks of age, whereas dexamethasone-exposed animals (*n* = 8) were 22.8 ± 0.5 weeks of

age ($P > 0.75$, control compared with dexamethasone-exposed). Animal weights were also similar between groups (control, 51.1 ± 1.8 kg; dexamethasone-exposed, 48.0 ± 3.1 kg), although females in each group tended to be smaller.

Rates of oxygen consumption were measured in the cardiac mitochondria isolated from the control and dexamethasone-exposed sheep using both Complex I-(glutamate and malate) and Complex II (succinate)-dependent substrates in the presence of ADP (State 3) and after ADP depletion (State 4). As shown in Table 1, no significant differences were seen between control and dexamethasone-exposed cardiac mitochondria in either State 3 or State 4 oxygen-consumption rates.

Mitochondrial H_2O_2 production

Production of ROS from Complexes I and III in both mitochondria and mitochondrial membranes was investigated with the use of various substrates and complex inhibitors as demonstrated in the schematic diagram shown in Figure 1. When control and dexamethasone-exposed groups were compared, significant differences in the rate of H_2O_2 production were found when the cardiac mitochondria were incubated in the presence of glutamate and malate with rotenone and rotenone plus the uncoupler FCCP, or succinate with rotenone and rotenone plus FCCP (Figure 2). When female and male animals were considered separately by two-way ANOVA, it was found that the primary source of the differences between control and dexamethasone-exposed sheep was due to differences in the female cardiac mitochondria. No effect of gender between control groups was identified by two-way ANOVA.

Important information about mitochondrial H_2O_2 production was also obtained by examining the data from all of the study groups that are shown in Figure 2. In the presence of glutamate/malate, addition of rotenone had the expected effect of increasing H_2O_2 production. Furthermore, as has been demonstrated previously [28], rotenone decreased H_2O_2 production by succinate through its blockade of 'reverse' electron flow from Complex II (succinate dehydrogenase) to Complex I. Consistent with the theory that Complex III is an important site of $O_2^{\bullet-}$ production [11], inhibition of Complex III with antimycin A increased H_2O_2 production whether glutamate/malate or succinate were used as substrates. Addition of the uncoupler FCCP decreased H_2O_2 production whether glutamate/malate or succinate were present, suggesting that decreased mitochondrial membrane potential and complex oxidation in the presence of the uncoupler decreased electron leak [14]. With succinate as the substrate, the decrease in H_2O_2 production with the addition of FCCP was particularly marked, probably due to diminished 'reverse' movement of electrons to Complex I (Figure 2). Interestingly, the greatest levels of H_2O_2 production were seen when succinate was added as a substrate, the mitochondria were uncoupled with FCCP and Complex III was inhibited with antimycin A.

Mitochondrial membrane H_2O_2 production

Mitochondrial membranes were prepared so that comparisons of H_2O_2 production could be made between control and dexamethasone-exposed animals in the absence of detoxification of the $O_2^{\bullet-}$ species that occurs in the mitochondrial matrix of intact mitochondria. When studying the mitochondrial membranes, NADH was used to deliver reducing equivalents to Complex I since the mitochondrial membranes cannot utilize glutamate/ malate. In the mitochondrial membranes, the Complex-I-dependent differences in H_2O_2 production that were found in intact cardiac mitochondria were lost, no differences were found with NADH or NADH plus rotenone in the mitochondrial membranes (Figure 3). Mitochondrial membranes prepared from dexamethasone-exposed animals had a small, but significant, increase in H_2O_2 production when succinate and rotenone were added to the particles.

Preparation of mitochondrial membranes resulted in greater levels of H₂O₂ production compared with intact mitochondria when NADH dehydrogenase (Complex I)-driven ROS production was considered probably due to decreased detoxification of O₂^{•-} in the mitochondrial matrix. This was evident by the nearly 3-fold increase in levels of H₂O₂ production seen in the mitochondrial membranes studied in the presence of NADH compared with the intact mitochondria incubated with glutamate/malate (intact mitochondria are impermeable to NADH). The theory that O₂^{•-} produced by the Q_i centre of Complex III is directed outside the mitochondria is supported by the similar levels of H₂O₂ production that were found between mitochondrial membranes and intact mitochondria when succinate and antimycin A were included.

Complex I–III activity and protein levels

The maximal rate of cytochrome *c* oxidation in mitochondrial membranes was used to determine the maximal flux rates through Complexes I and III of the electron-transport chain. Rates of cytochrome *c* oxidation were similar between control and dexamethasone-exposed cardiac mitochondria (Figure 4).

To support the Complex I–III enzyme activity data, steady-state protein levels of the 39 kDa subunit of Complex I and the core 2 subunit of Complex III were measured by immunoblot analysis. Protein levels of both of the Complex I and III subunits were not different between control and dexamethasone-exposed sheep (Figure 5).

Antioxidant enzyme pathways

Several pathways exist within the mitochondria to detoxify O₂^{•-} species [29]. Mn-SOD catalyses the conversion of O₂^{•-} into H₂O₂. As shown in Figure 4, Mn-SOD activity tended to be lower in the dexamethasone-exposed cardiac mitochondria, although the difference did not reach statistical significance (*P* = 0.17). To determine whether there were absolute differences in Mn-SOD protein levels, immunoblot analysis was performed. Steady-state myocardial Mn-SOD protein levels were not different between control and dexamethasone-exposed animals (Figure 5).

Both GPx and catalase convert H₂O₂ into water. Interestingly, catalase activity was significantly increased in dexamethasone-exposed cardiac mitochondria compared with controls (Figure 4). No gender-specific differences were identified by two-way ANOVA. No significant changes in GPx activities were found between groups.

DISCUSSION

The concept of fetal or developmental programming of disease is emerging as an important public health issue. As the scope of this problem expands beyond the experimental realm and begins to impact public health policy, the mechanisms by which exposure to an adverse intrauterine environment contributes to an increased risk for cardiovascular disease will be critical to delineate. In the present study, the sheep model of developmental programming was used that imposes a well-defined insult on the developing fetus with exposure to maternal administration of dexamethasone for 2 days in the first trimester of gestation [5,30]. Postnatally, animals exposed to dexamethasone during this critical period develop elevated blood pressure [31], changes in sympathetic tone [32] and changes in vessel reactivity [15,16]. It is likely that some of these changes are mediated by altered ROS production [6]. Measurements of cardiac mitochondrial ROS produced and released as H₂O₂ were performed in the present study using mitochondria isolated from the control and dexamethasone-exposed sheep. Increased rates of H₂O₂ release were found in intact mitochondria from dexamethasone-exposed sheep from Complex I, which directs O₂^{•-}

production into the mitochondrial matrix [33]. When mitochondrial membranes were studied, differences among the programmed and control groups were not found. This suggested that maximal rates of $O_2^{\bullet-}$ production by Complexes I and III were not affected by exposure to an adverse *in utero* environment. Of further interest was the finding that the activity of catalase, an enzyme in the mitochondrial matrix that detoxifies H_2O_2 to water, was increased in the programmed mitochondria. Thus, despite an enhanced ability to breakdown H_2O_2 produced in the mitochondrial matrix space, these same animals produced significantly greater amounts of extramitochondrial H_2O_2 . The finding that Mn-SOD was not different among the groups indicated that the rate of formation of H_2O_2 from $O_2^{\bullet-}$ did not have an impact upon the enhanced rate of H_2O_2 release from the mitochondrial matrix space. Taken together, these findings indicate that the rate at which H_2O_2 was released from the mitochondrial matrix space was increased in the programmed animals.

Sex-specific differences in developmental programming have been described in several models [34,35]. Thus it is not surprising that we found differences in mitochondrial H_2O_2 production that were prominent in females. In the sheep model used in the present study, gender differences due to exposure to an adverse intrauterine environment have not been published previously. Although the mechanism of the gender differences identified in the present study is not known, the current findings indicate that careful review of gender-specific data is warranted.

Mitochondrial ROS production

Mitochondrial production of ROS has been recognized for several decades [13,36]. Early studies identified that $O_2^{\bullet-}$ was generated at both Complexes I and III in the electron-transport chain, although their relative contribution to total mitochondrial ROS production has been disputed [11,13,27]. In the present study, generation of ROS at Complex I in intact mitochondria was explored using glutamate and malate as substrates in the presence and absence of the Complex I inhibitor rotenone (see Figure 1). By binding at the downstream end of Complex I [37], rotenone causes reduction in the upstream components of the complex, which enhances electron leak into the mitochondrial matrix space where they are subject to matrix detoxification pathways [11,13].

Consistent with previous studies, we found that backward electron flow derived from the Complex II substrate succinate is an important source of mitochondrial ROS production [38]. This was evident from the high rates of H_2O_2 production that were found in mitochondria incubated with succinate as the substrate (Figure 2). Furthermore, the rate of H_2O_2 release decreased significantly when rotenone was added to succinate. This latter finding also supports the theory that rotenone inhibits Complex I at a location downstream from the site of ROS production.

The studies with the uncoupler FCCP in the intact mitochondria provided insight into the role the mitochondrial membrane potential, complex redox state and flux through the electron-transport chain play in the generation of $O_2^{\bullet-}$. Several studies have demonstrated that a decrease in mitochondrial ROS production occurs with a decrease in mitochondrial membrane potential [39,40]. Similarly, early studies found that mitochondria operating in State 3 (excess substrate and ADP) generate low levels of $O_2^{\bullet-}$ [13,41]. In the present study, the lowest rates of H_2O_2 production were found when FCCP was added. The most striking decrease occurred when FCCP was added to succinate. This suggested that the addition of FCCP to succinate not only essentially diminished the backward flow of electrons into Complex I, but also decreased $O_2^{\bullet-}$ production by Complex III.

Superoxide that is produced by Complex I and the Q_i centre of Complex III is directed into the mitochondrial matrix. In order for these ROS to be measured in the extramitochondrial

space using the AmplexRed reagent, $O_2^{\bullet-}$ must be exported from the mitochondria and converted into H_2O_2 or be converted into H_2O_2 within the matrix space and diffuse outside the mitochondria. Although H_2O_2 freely diffuses across cell membranes, $O_2^{\bullet-}$ does not. It has been suggested that $O_2^{\bullet-}$ can be released by mitochondria through a channel activated by $O_2^{\bullet-}$ itself (so-called ROS-induced ROS release), although this has not been extensively studied [42,43]. If $O_2^{\bullet-}$ was released in appreciable amounts from the mitochondria in the present study, conversion into H_2O_2 was spontaneous and rapid since initial studies showed that addition of SOD to the reaction medium did not affect the measured rate of H_2O_2 production.

Antioxidant pathways

Several antioxidant pathways exist within the mitochondria to limit damage to the organelle by $O_2^{\bullet-}$. The initial detoxification step is to convert $O_2^{\bullet-}$ into H_2O_2 in a reaction catalysed by Mn-SOD [44]. Given the significant differences in H_2O_2 production between control and programmed animals when Complex-I-driven $O_2^{\bullet-}$ production was studied in the intact mitochondria, it was somewhat surprising that Mn-SOD activity was not different between the groups. The small, but non-significant, decrease in Mn-SOD activity in the dexamethasone-exposed animals (Figure 4) may have resulted in an increase in available $O_2^{\bullet-}$ to diffuse out of the mitochondria and convert into H_2O_2 for measurement by the AmplexRed reagent. Direct measurement of mitochondrial matrix $O_2^{\bullet-}$ in intact mitochondria would be useful in future studies to define changes in matrix detoxification by Mn-SOD.

Two reactions exist within the mitochondria to convert H_2O_2 into water, reactions catalysed by GPx and catalase [45–47]. Interestingly, a significant increase in catalase activity was seen in mitochondria from the dexamethasone-exposed animals (Figure 4). An increase in catalase activity has been demonstrated when cells were placed under oxidative stress [48]. In the present study, an increase in catalase activity would potentially limit the amount of H_2O_2 available for release from the mitochondria and may reflect an adaptive response of the dexamethasone-exposed animals. Without the significant increase in catalase activity, it is possible that measured levels of H_2O_2 production from Complex I in the dexamethasone-exposed mitochondria would have been even greater.

Limitations

The sheep model of developmental programming used in the present study provides a very specific temporal insult to the fetus. The physiological basis of this intervention, however, is not completely defined. It is possible that direct actions of the steroid result in the programming effect, although it is likely that downstream pathways result in epigenetic changes that permanently alter gene expression [49]. Although phenotypically similar, the mechanistic link of the sheep model to human developmental programming of adult diseases that is related to low birthweight is not defined at this time, which may limit the clinical applicability of our results.

The age at which the dexamethasone-exposed and control animals were studied may also have an impact on the results of the present study. By 22–23 weeks of age (the age used in the present study), the dexamethasone-exposed sheep are beginning to demonstrate hypertension, although no increase in left ventricular mass has been found in previous studies at this time point [15]. Thus it is possible that the increase in H_2O_2 production that was seen reflects a response to the hypertension and not a primary developmental programming event. Further studies at earlier time points will be needed to define when the cardiac mitochondria from dexamethasone-exposed sheep start to generate increased ROS.

The exact mechanism by which increased H₂O₂ production occurs in the dexamethasone-exposed animals has not been completely defined. Previous studies have correlated increased mitochondrial ROS production with diminished complex activity [12,50], which was not seen in the present study. Lastly, mitochondria were studied in State 4, which does not reflect their typical *in vivo* state where ADP and substrates are available. However, the results of the present study may be relevant to *in vivo* mitochondrial ROS production during times when substrate availability is limited, such as during myocardial ischaemia when reduction of the electron-transport chain complexes would be expected to occur.

Perspectives

The mechanisms by which exposure to an adverse intrauterine environment results in adult disease are not known. The present study identified that, in the sheep model of developmental programming, exposed offspring release significantly more mitochondrial H₂O₂ than their non-programmed counterparts. Although the relationship of this finding to the risk of developing hypertension and cardiovascular disease was not explored in the present study, the importance of ROS in cardiac morbidity is well known. Defining the impact of altered mitochondrial ROS production on the cardiovascular system in developmental programming and determining whether programming occurs in mitochondria from other organs (e.g. endothelium) will be essential as future therapies are defined.

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Abbreviations

FCCP	carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazine
GPx	glutathione peroxidase
ROS	reactive oxygen species
SOD	superoxide dismutase
Mn-SOD	manganese SOD

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Figure 1. Schematic diagram of the electron-transport chain

A section of the inner mitochondrial membrane is shown with Complexes I—IV. Arrows identify the flow of electrons within the electron-transport chain. $O_2^{\bullet-}$ produced from Complex I and the Q_i center of Complex III is directed into the mitochondrial matrix, whereas $O_2^{\bullet-}$ generated at the Q_o centre is released into the inter membrane space. The inhibitors rotenone and antimycin A and their sites of inhibition are shown. Cyt. c, cytochrome c.

Figure 2. H₂O₂ production by intact sheep left ventricular mitochondria

Intact mitochondria were isolated from the left ventricle of 4-month-old sheep from either animals programmed *in utero* with dexamethasone (Dex-Exposed) or saline-exposed (Control). Intact cardiac mitochondria were incubated with substrates oxidized by Complex I [glutamate/malate (GM)] or Complex II [succinate (Succ)], with or without inhibitors to Complex I [rotenone (Rot)] or Complex III [antimycin A (Ant A)] and/or the uncoupler FCCP. Production of H₂O₂ was detected with the fluorogenic indicator AmplexRed. Values are means $\pm$ S.E.M. ($n = 9$ for control and $n = 8$ for dexamethasone-exposed). * $P < 0.05$, dexamethasone-exposed compared with the control.

Figure 3. H₂O₂ production by mitochondrial membranes

Mitochondrial membranes were prepared from mitochondria isolated from the left ventricle of 4-month-old sheep from either animals programmed *in utero* with dexamethasone (Dex-exposed) or saline-exposed (Control). Mitochondrial membranes were incubated with substrates oxidized by Complex I (NADH) or Complex II [succinate (Succ)], with or without inhibitors to Complex I [rotenone (Rot)] or Complex III [antimycin (Ant A)]. Production of H₂O₂ was detected with the fluorogenic indicator AmplexRed. Values are means $\pm$ S.E.M. ($n = 9$ for control and $n = 8$ for dexamethasone-exposed). * $P < 0.05$, dexamethasone-exposed compared with control.

Figure 4. Complex I-III and antioxidant enzyme activities measured in mitochondria from control and dexamethasone-exposed (Dex-Exposed) animals

Mitochondrial membranes were used to determine Complex I-III activity which was measured based on the rate of cytochrome *c* reduction in μM cytochrome *c* $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. Intact mitochondria were used for assays of the antioxidant enzymes. Mn-SOD, GPx and catalase were measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. ($n = 9$ for control and $n = 8$ for dexamethasone-exposed). * $P < 0.05$, dexamethasone-exposed compared with control measured by two-way ANOVA. mU, milli-units.

Figure 5. Protein levels of Complex I, Complex III and Mn-SOD in control and dexamethasone-exposed (Dex-Exposed) sheep left ventricular myocardium

Steady-state protein levels of the 39 kDa subunit of Complex I (Complex I), the core 2 subunit of Complex III (Complex III) and Mn-SOD in ovine left ventricular myocardium from animals programmed *in utero* with dexamethasone (D) or saline-exposed (C).

Myocardial protein was isolated from ovine left ventricular free wall and separated by SDS/PAGE. Membranes were probed with specific antibodies followed by detection of bound antibody with IR-labelled secondary antibodies. Sizes of the observed proteins based on simultaneously run molecular-mass markers were: Complex I, 42 kDa; Complex III, 48 kDa; and Mn-SOD, 25 kDa. Values are means $\pm$ S.E.M. ($n = 12$ for control and $n = 13$ for dexamethasone-exposed).

Table 1
Oxygen consumption rates of intact left ventricular mitochondria isolated from control and dexamethasone-exposed animals in the presence of glutamate/malate or succinate

Values are means $\pm$ S.E.M. The rate of oxygen consumption is given as $\text{nM O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein.

Mitochondrial state	Control ($n = 9$)	Dexamethasone-exposed ($n = 8$)
State 3: glutamate/malate	279 ± 36	204 ± 33
State 3: succinate	163 ± 21	145 ± 18
State 4: glutamate/malate	18 ± 6	36 ± 8
State 4: succinate	38 ± 5	30 ± 7